Seed Genomics

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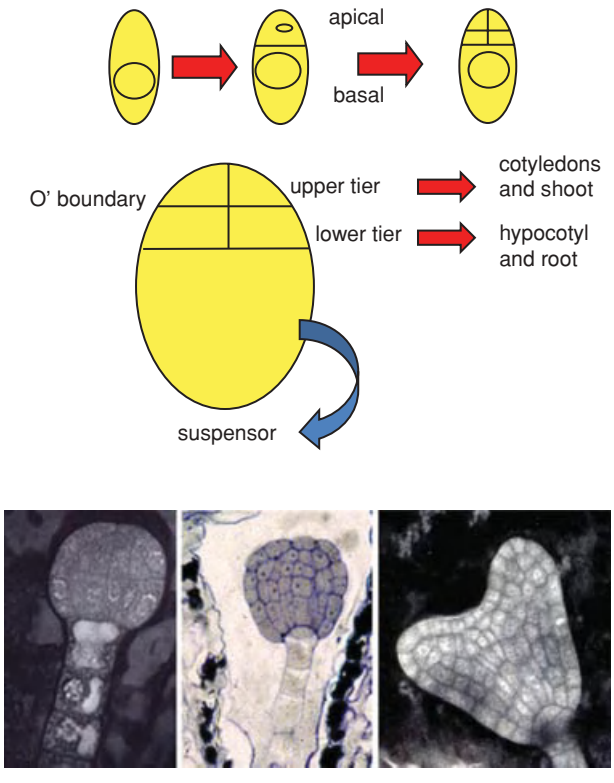
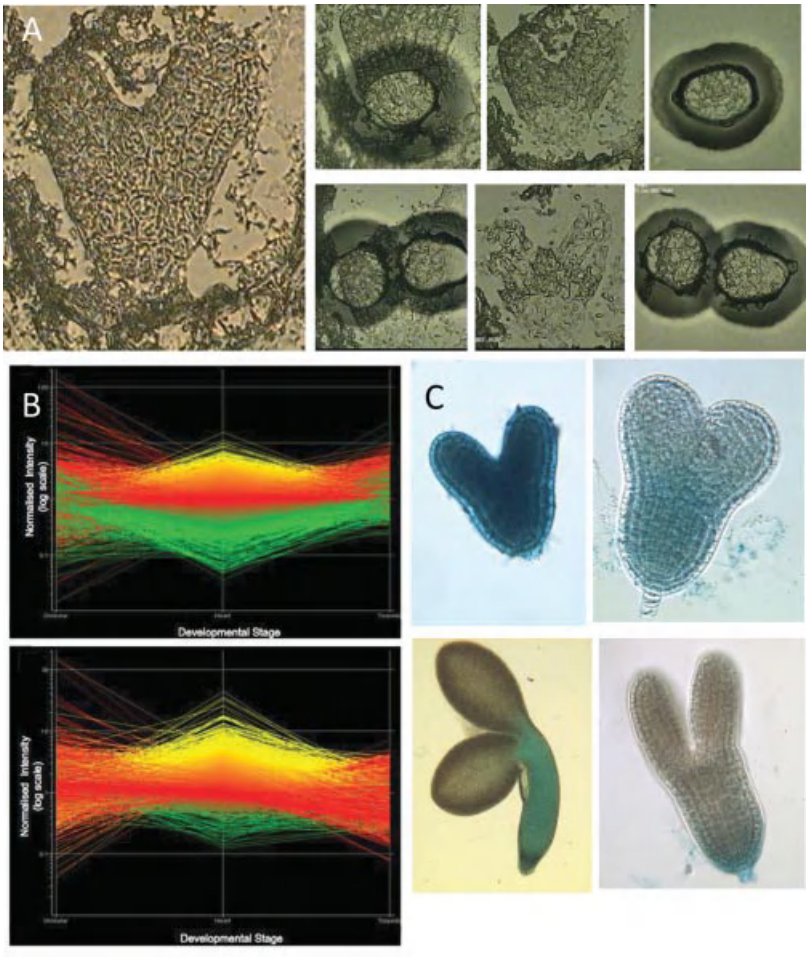


Plate 2.1 *Upper panel,* Diagrammatic representation of early events in *Arabidopsis* embryogenesis. The zygote undergoes an asymmetrical transverse division to form a small apical cell and a larger, more vacuolated basal cell. The basal cell divides to form the suspensor, which the apical cell divides transversely to form upper and lower tiers that develop into the cotyledons and shoot apical meristem and the hypocotyl and root. *Lower panel,* Developing globular *(left, center)* and heart *(right)* stage embryos of *Arabidopsis,* showing the upper cells of the suspensor.



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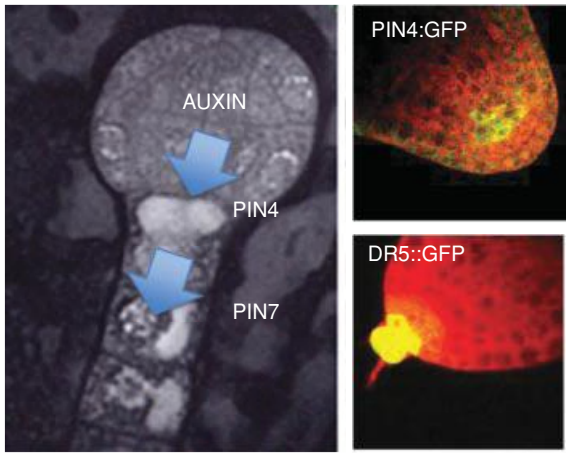


Plate 2.3 Auxin is translocated in the developing globular embryo from the apical region to the hypophysis via PIN4 and then from the hypophysis to the suspensor via PIN4 and PIN7. There is an auxin maximum (seen as DR5::GFP expression) at the basal region of the embryo.

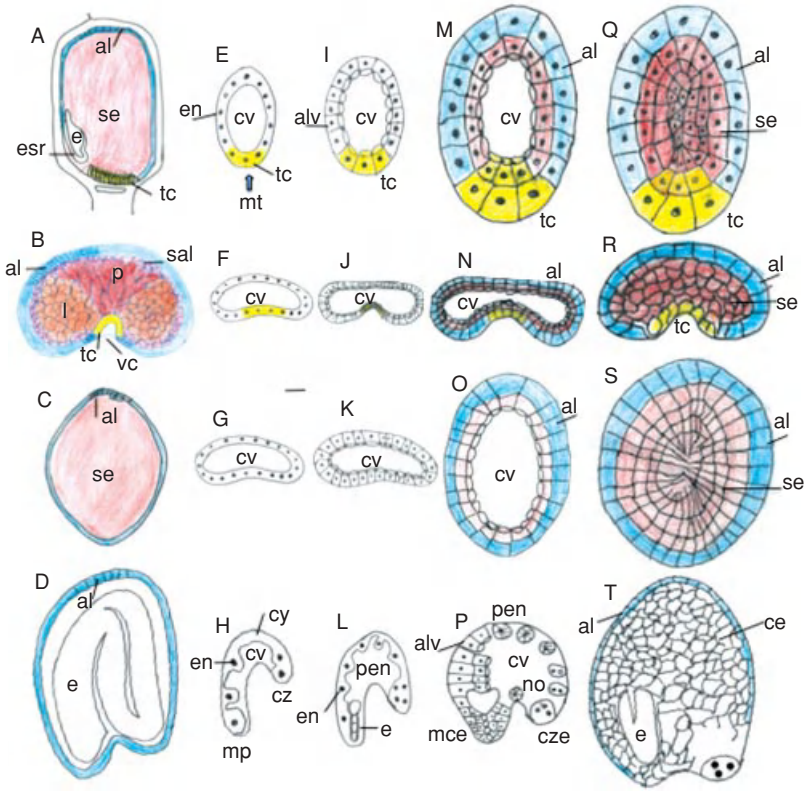


Plate 3.1 Overview of endosperm structure. *A–D*, Mature endosperm of maize *(A)*, barley *(B)*, rice *(C)*, and *Arabidopsis thaliana (D)*. al, aleurone; e, embryo; esr, embryo surrounding region; i, irregular starchy endosperm cells; ma, modified aleurone layer (transfer cells); p, prismatic cells; sal, subaleurone layer; se, starchy endosperm; tc, transfer cells; vc, ventral crease. *E–H*, Coenocytic stage endosperm of maize *(E)*, barley *(F)*, rice *(G)*, and *Arabidopsis thaliana (H)*. cv, central vacuole; tc, transfer cell region; *Meg1*, maternal transcript involved in induction of transfer cell fate specification; Mp, micropylar end; cz, chalazal end; en, endosperm nucleus; cy, endosperm cytoplasm. *I–L*, Alveolar stage of endosperm development of maize *(I)*, barley *(J)*, rice *(K)*, and *Arabidopsis thaliana (L)*. *M–P*, Cellularizing endosperm with one peripheral cell layer and inner alveoli in maize *(M)*, barley *(N)*, rice *(O)*, and *Arabidopsis thaliana (P)*. *Q–T*, Fully cellular endosperm of maize *(Q)*, barley *(R)*, rice *(S)*, and *Arabidopsis thaliana (T)*.

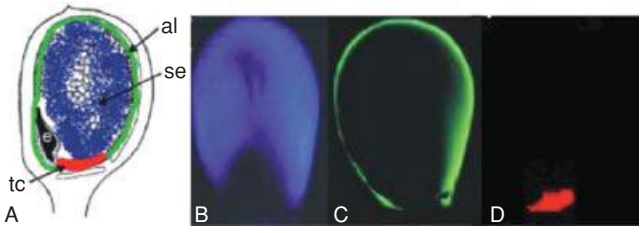
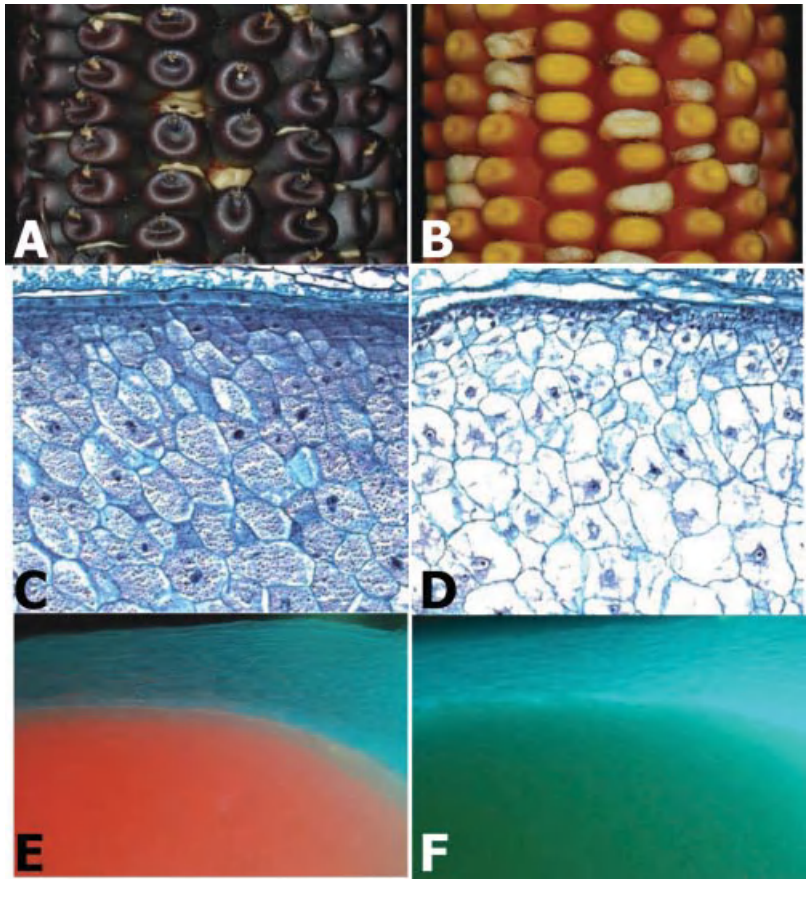


Plate 3.3 Kernel of "triple marked" maize endosperm. *A*, Diagram of the three major endosperm tissues. al, aleurone; e, embryo; se, starchy endosperm; tc, transfer cells. *B–D*, Expression of fluorescent cell-type markers in 12 DAP endosperm. Various cell-specific promoters are used to regulate expression of fluorescent proteins with differing spectral properties.B, γ-Zein:AmCyan expression fluoresces blue in the starchy endosperm. *C*, Ltp2:ZsYellow expression is specific for the aleurone layer. *D*, End1:DsRed expression marks transfer cells. (Modified from Gruis *et al.* [2006].)



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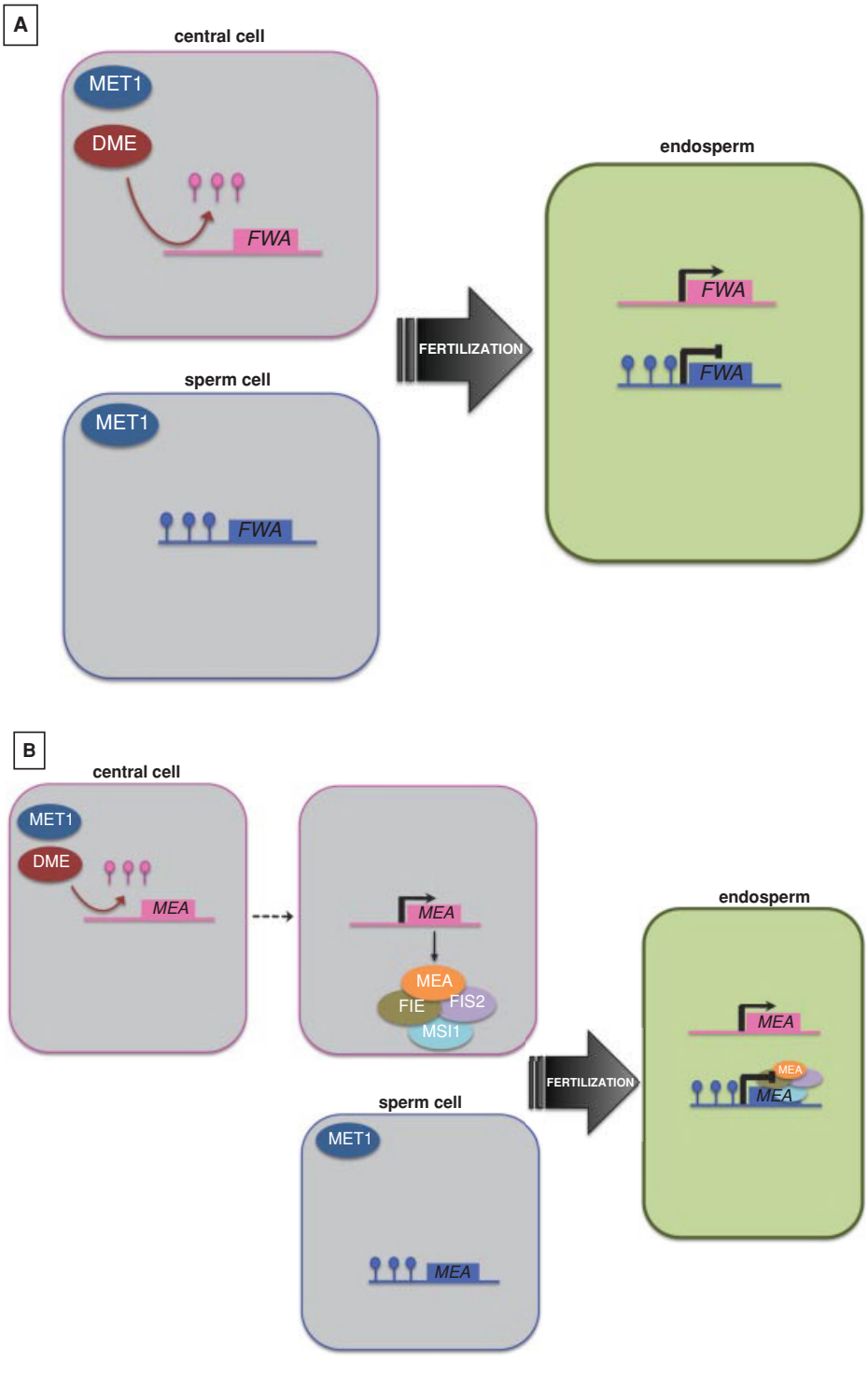
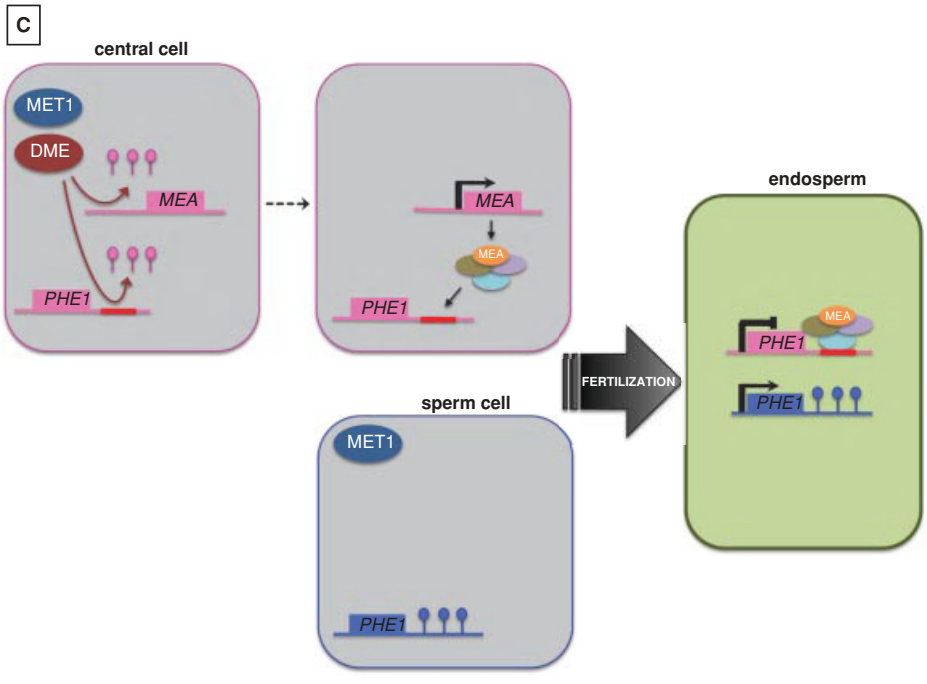


Plate 4.1 *A–C,* Models for *Arabidopsis* genomic imprinting: *FWA* model *(A)*, *MEA* model *(B)*, and *PHE1*-model *(C)*. "Lollipops" represent 5-methyl cytosines. The red bar on the maternal *PHE1* allele in represents the location of the 3′-tandem repeats targeted for DME-mediated DNA demethylation.



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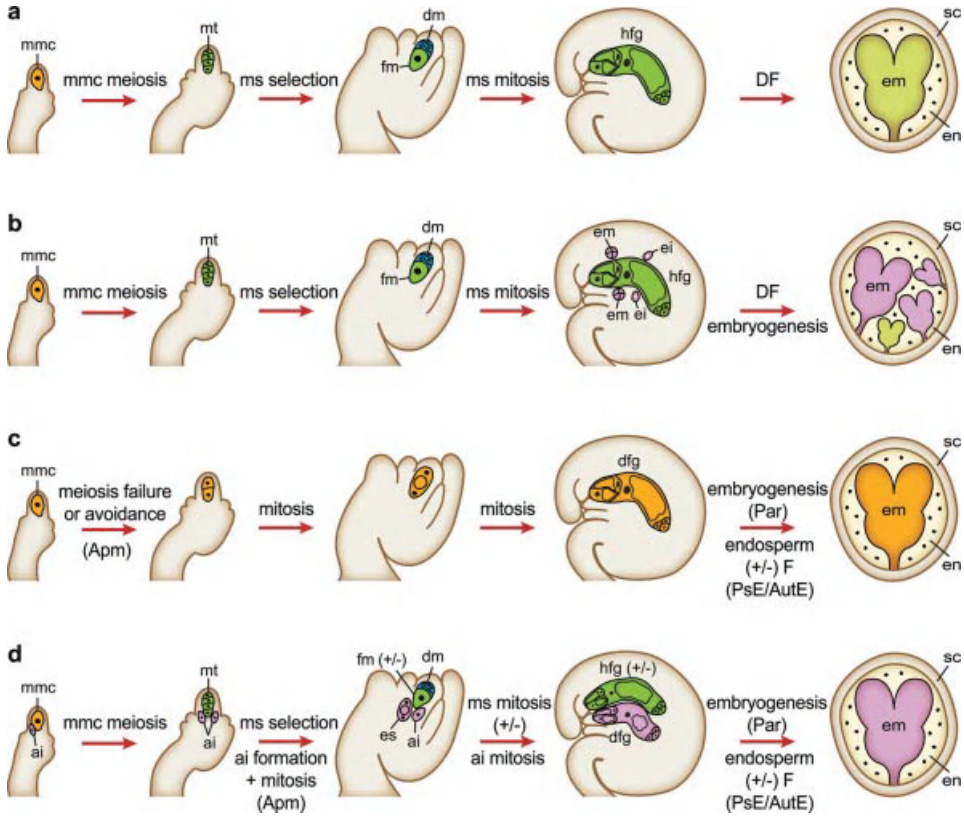

Plate 5.1 Seed development in sexual and apomictic species. *a,* Events of sexual seed formation. *b,* Events of sporophytic apomixis. *c,* Events of diplosporous apomixis and, *d,* aposporous apomixis, where endosperm formation may or may not require fertilization. In the case of apospory, haploid female gametophytes may coexist with the aposporous gametophyte, or degeneration of the haploid gametophyte may occur during the events of aposporous gametophyte development. See text for further details. ai, aposporous initial cells; Apm, apomeiosis; AutE; autonomous endosperm formation; DF, double fertilization; dfg, diploid female gametophyte; dm, degenerating megaspores; ei, embryo initial cell; em, embryo; en, endosperm; F, fertilization; fm, functional megaspore; hfg, haploid female gametophyte; mmc, megaspore mother cell; ms, megaspore; mt, meiotic tetrad; Par, parthenogenesis; PsE, pseudogamous endosperm formation which requires fertilization; sc, seed coat; (+/−), present or absent. (Modified from Drews and Koltunow, 2011.)

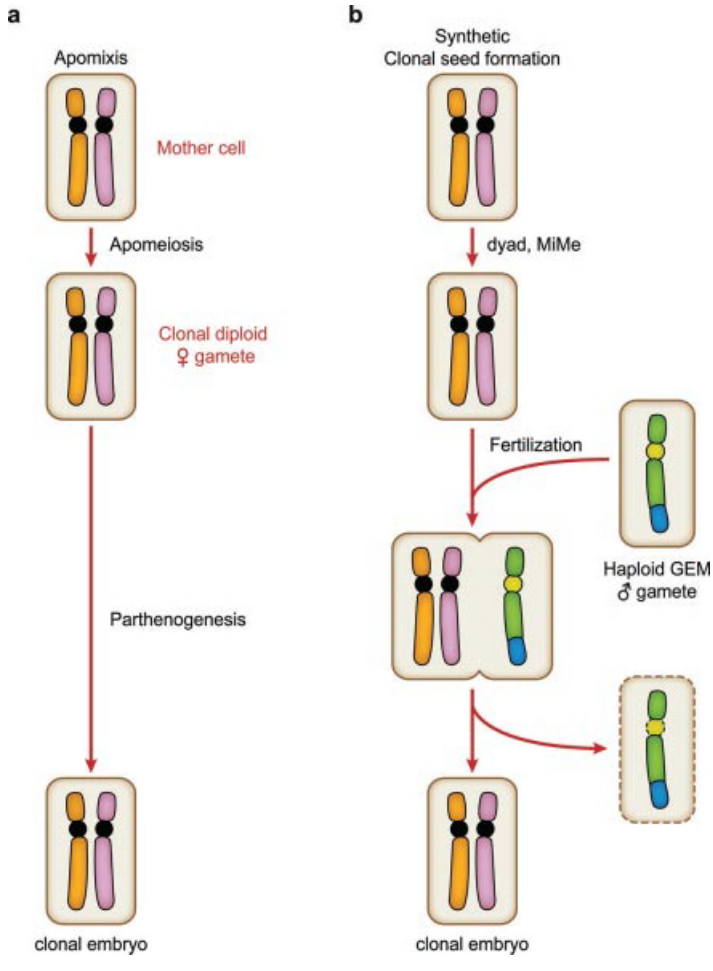

Plate 5.2 Natural and synthetic clonal reproduction through seed. *a,* In natural apomicts, clonal embryos with a maternal genotype develop without fertilization. *b,* Induction of clonal embryos in sexual plants as described by Marimuthu *et al.* (2011) involves combining mutants so that gametogenesis occurs without meiosis and subsequent fertilization with a parent whose chromosomes are modified to be eliminated after fertilization. (Modified from Marimuthu *et al.*, 2011.)

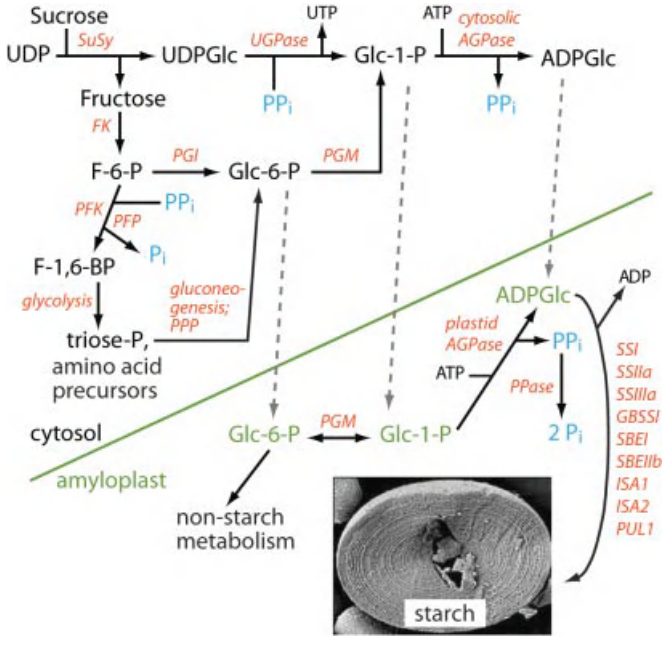

Plate 7.1 Outline of endosperm metabolism from sucrose to starch. *Gray lines* indicate transport from the cytosol into the amyloplast stroma. Glc-1-P is supplied either by UGPase in the "direct" path from sucrose or from metabolic conversion to triose phosphates and resynthesis back into hexose phosphate. Enzymes noted as being involved in starch biosynthesis have been identified by genetic analyses; this does not preclude the involvement of additional isoforms. SuSy, sucrose synthase; UGPase, UDP glucose pyrophosphorylase; FK, fructokinase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; PFK, phosphofructokinase; PFP, pyrophosphate-dependent phosphofructokinase; PPP, pentose phosphate pathway; PPase, pyrophosphatase; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-bisphosphate.

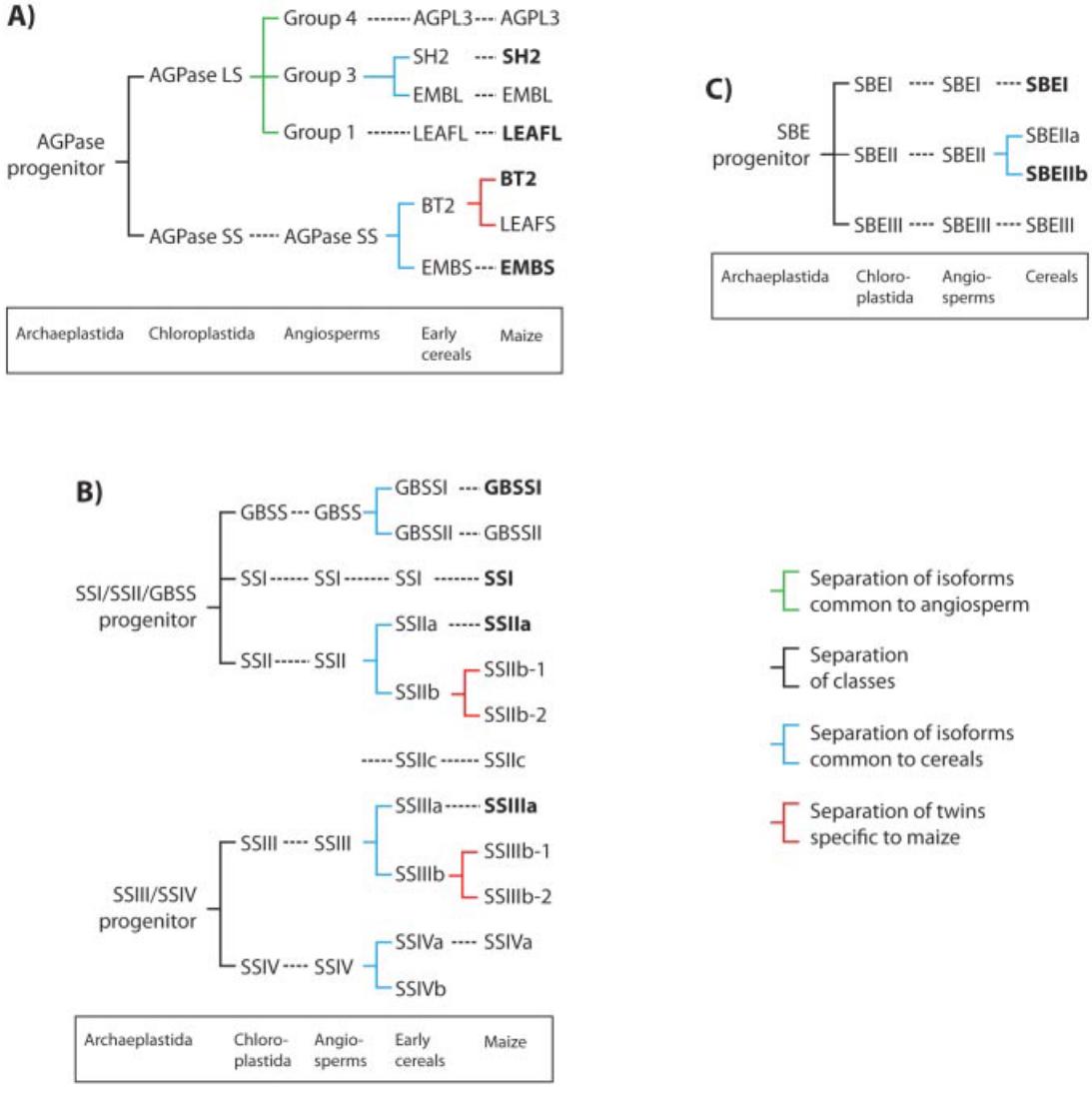

Plate 7.2 Proposed evolution of maize starch biosynthetic genes. Enzymes in bold text have been shown by genetic analysis to be involved in endosperm starch biosynthesis. Only lineages that lead to genes extant in maize are shown. *A)* AGPase. A single gene in the Archaeplastida progenitor, derived from a cyanobacterium, duplicated to form AGPase LS and AGPase SS that are conserved in all chloroplast-containing species. An angiosperm progenitor underwent further duplications to generate a minimum of three isoforms of AGPase LS and one of AGPase SS before separation of monocots and eudicots. In the Graminacea, a whole-genome duplication resulted in two isoforms of the group 3 AGPase LS and two isoforms of AGPase SS. A whole-genome duplication generated the duplicate gene encoding LEAFS that is present in maize but not in rice. *B)* SS. Two genes were present in the primoridal Archaeplastida progenitor before divergence of plastid-containing organisms. At the establishment of the Chloroplastida, these had duplicated to form five SS classes, each of which is extant in all green algae and land plants. In the Graminacea, a whole-genome duplication resulted in isoforms of GBSS, SSII, and SSIII, each of which is present in maize and rice. SSIV was also duplicated at this stage, but one copy was not retained in maize. SSIIc could have originated either in an ancestor common to both monocots and eudicots or after that division. A whole-genome duplication generated twin genes for SSIIb and SSIIIb that are present in maize but not in rice. *C)* SBE. Three classes of SBE had been formed before divergence of green algae and land plants by duplication of an ancestral gene. In general, all three classes are conserved in Chloroplastida species, although at least one exception is known. The ancestral whole-genome duplication in the Graminaceae gave rise to two isoforms of the SBE II class, both of which are present in maize and rice. (Adapted from Rosti and Denyer [2007]; Deschamps *et al.* [2008]; Georgelis *et al.* [2008]; and Yan *et al.* [2009a, 2009b].)

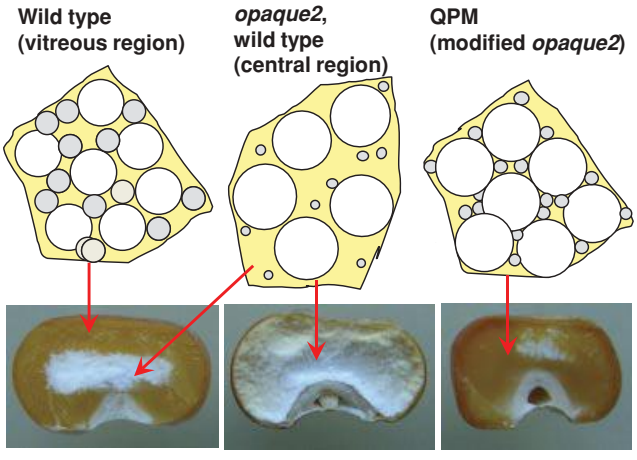

Plate 8.2 Diagram of protein body and starch grain interaction in vitreous endosperm formation. Individual cells of developing endosperm are represented with the relative size and abundance of starch grains (white spheres) and zein protein bodies (gray spheres) that are thought to result in vitreous or opaque endosperm in normal, *opaque2,* and modified *opaque2* (QPM) kernels.

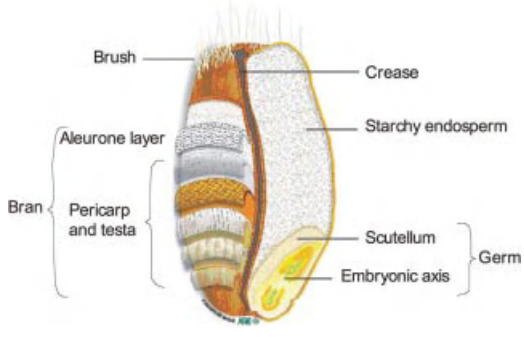

Plate 9.1 Component tissues of wheat grain. (From Surget and Barron [2005], with permission.)

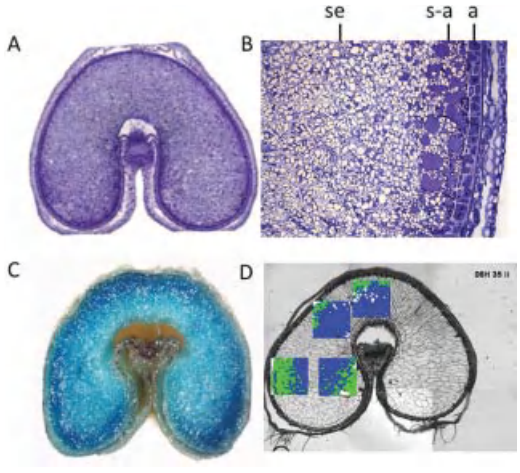

Plate 9.2 Transverse sections of developing caryopses of wheat, showing gradients in composition in the endosperm. *A* and *B,* Low-magnification and high-magnification images of a transverse section of the whole caryopsis at about 28 days after anthesis, stained with toluidine blue to show protein. Aleurone *(a)* and subaleurone *(s-a)* cells are rich in protein, and central starchy endosperm *(se)* cells are rich in starch granules (unstained). *C,* Expression of low-molecular-weight glutein subunit-promoter-GUS fusion construct in a developing endosperm, showing high expression in the subaleurone and outer starchy endosperm cells. *D,* Overlaying of Fourier-transform infra-red microspectroscopy images onto a section of cell wall only at 35 days after anthesis. The images are colored to show the distributions of high-substitution (shown in blue) and low-substitution (shown in green) forms of arabinoxylan in the cell wall. (*A* and *B,* Courtesy Cristina Sanchez-Gritsch and Paola Tosi, Rothamsted Research; *C,* courtesy Caroline Sparks and Huw Jones, Rothamsted Research; *D,* from Toole *et al.* [2010] with permission.)

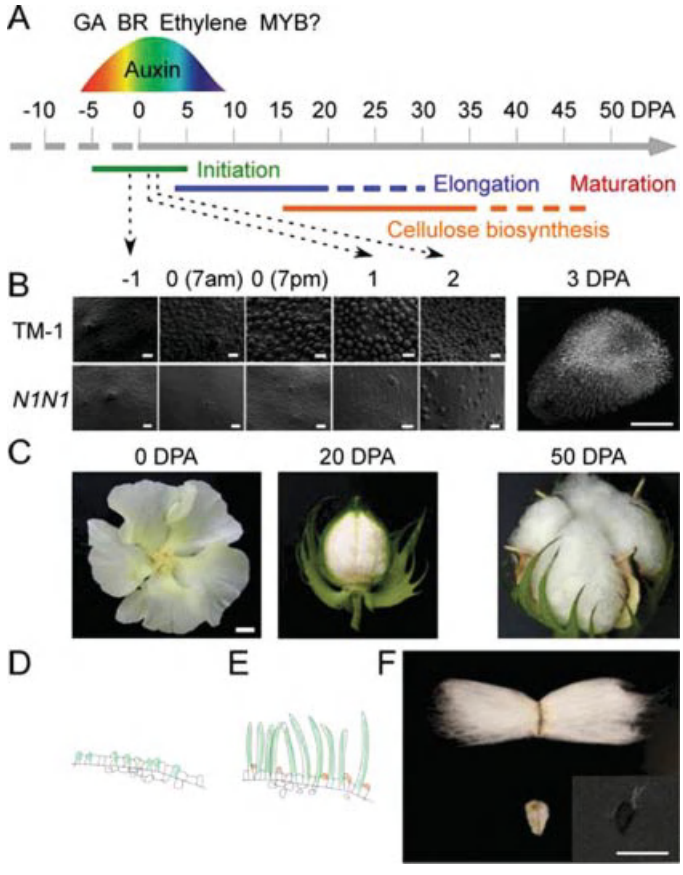

Plate 11.1 Cotton fiber development. *A,* Continuous and overlapping stages of cotton fiber development, including fiber cell initiation, elongation, cellulose (secondary cell wall) biosynthesis, and maturation. During elongation and maturation stages, cellulose biosynthesis is the dominant cellular metabolic process. (Secondary cell wall accumulation starts by depositing the cellulose under the primary cell wall after 15 DPA.) Regulation of phytohormones, including auxin, brassinosteroid (BR), and ethylene and transcription regulators, including MYB proteins, is implicated in cotton fiber cell initiation. *B,* Scanning electron microscope images of cotton fiber initiation processes. TM-1, *G. hirsutum* L. TM-1; N1N1, naked seed mutant. The ovules were collected at −1, 0 (7 a.m. and 7 p.m.), and 1 and 2 days after anthesis (0 = on the day of anthesis). Bar = 20 μm; bar = 100 μm for TM-1 ovule at 3 DPA. *C,* TM-1 cotton flower on 0 DPA, boll on 20 DPA and 50 DPA. *D,* Diagram of cotton fiber lint cell initiation on 0 DPA (green). *E,* Diagram of fuzz fiber cell initiation on 5–7 DPA (orange) along with lint fiber (green). F, Mature cotton lint and fuzz fiber on TM-1 seed. *Inset,* Mature seed of the fiberless mutant *XuFL.*

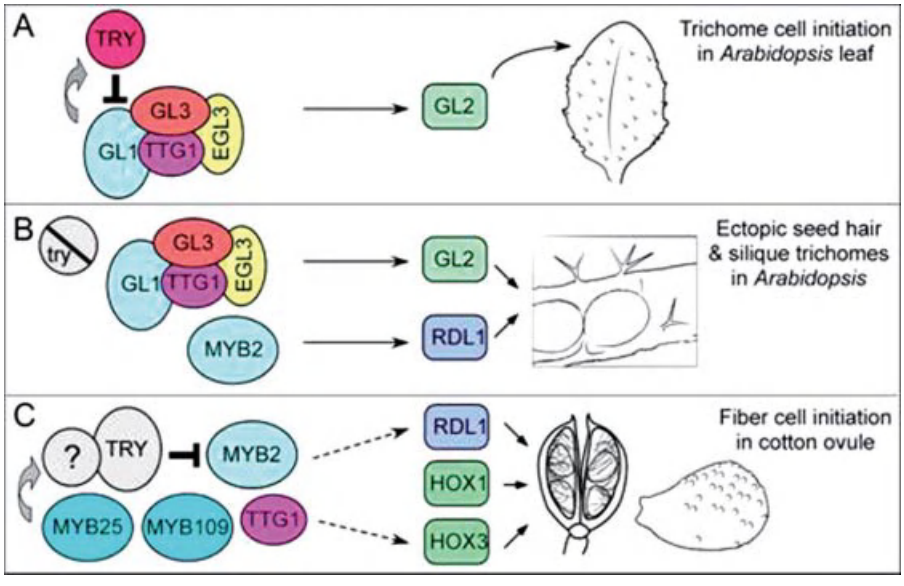

Plate 11.2 Models for leaf trichome and seed hair development. *A,* The trichome initiation complex consisting of GL1, GL3, EGL3, TTG1, and a negative regulator TRY promotes GL2 expression, leading to initiation of leaf trichomes in *Arabidopsis thaliana*. *B,* Cotton fiber genes, including *MYB2* and *RDL1,* stimulate the development of ectopic seed hairs and silique trichomes in *A. thaliana*. *C,* Multiple transcription factors identified in cotton fibers are shown to play roles in the development of seed hair and fiber in cotton. (From Guan *et al.* [2011].)

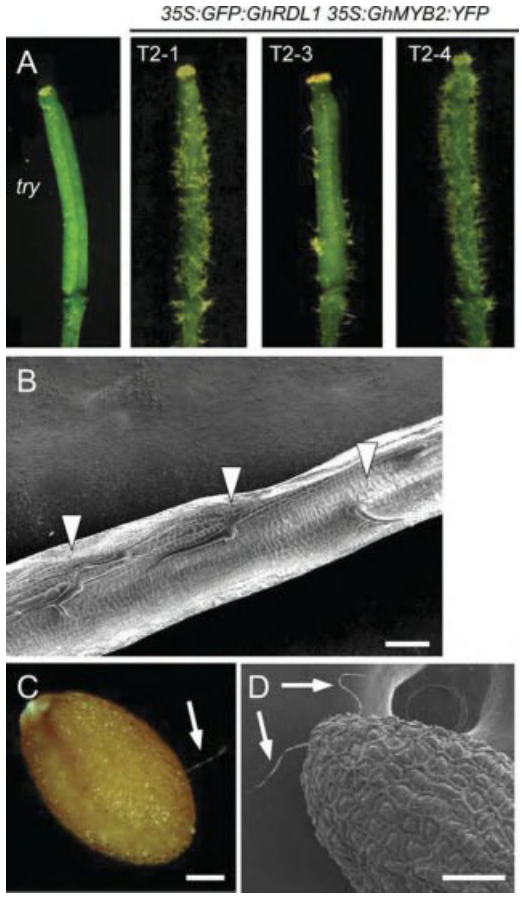

Plate 11.3 Development of ectopic trichomes inside and outside of siliques in transgenic *Arabidopsis thaliana* plants overexpressing *GhMYB2* and *GhRDL1*. *A,* Ectopic trichomes outside of siliques in transgenic *A. thaliana* plants. *B,* Ectopic trichomes inside of siliques *(arrows)* in transgenic *A. thaliana* plants. *C* and *D,* Seed hair observed in transgenic *A. thaliana* seeds under light microscope *(C)* and Scanning electron microscope *(D)*. (From Guan *et al.*, [2011].)

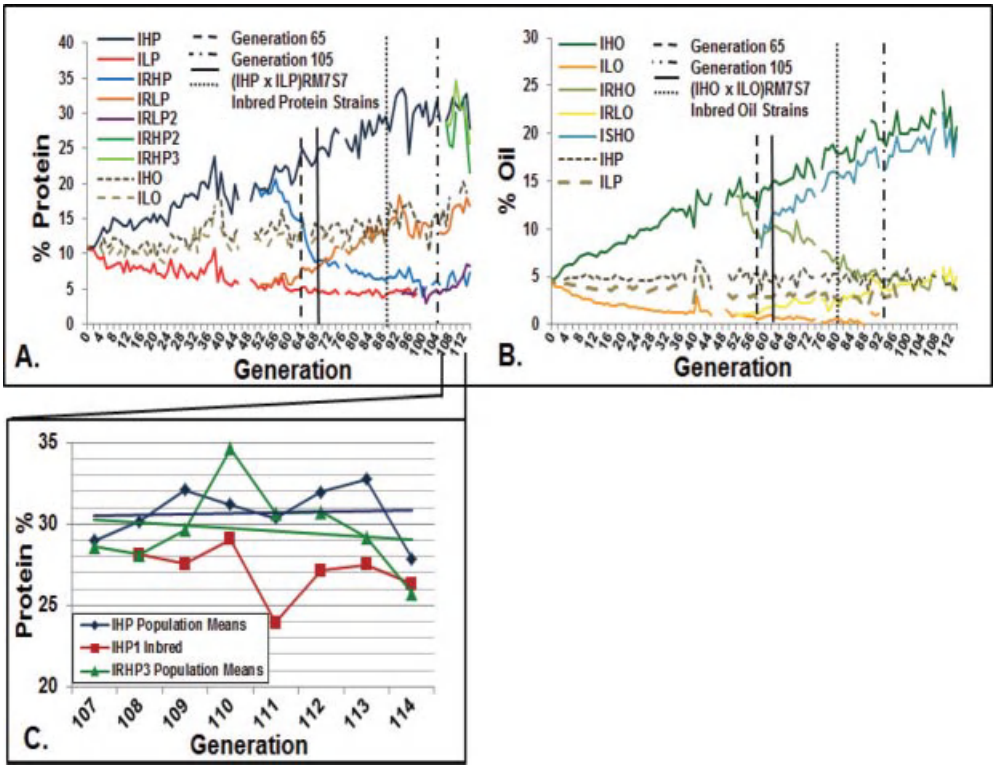

Plate 12.1 *A* and *B,* Selection responses in the Illinois Protein Strains *(A)* and Illinois Oil Strains *(B)*. Protein concentrations for IHO and ILO are plotted as *dashed lines* in *(A)*, and oil concentrations for IHP and ILP are plotted as *dashed lines* in *(B)*. The vertical lines highlight important generations of the experiment: generation 65, for which oldest seed is still available; generation 70, from which QTL mapping populations have been derived from crosses of selected strains; generation 90, as a source of inbred lines derived from the Illinois Protein Strains; and generation 105 from which inbred lines for the Illinois Oil strains are being produced. *C,* Expanded view of the last eight cycles of selection response in IHP and Illinois Reverse High Protein 3 compared with IHP1 inbred line.

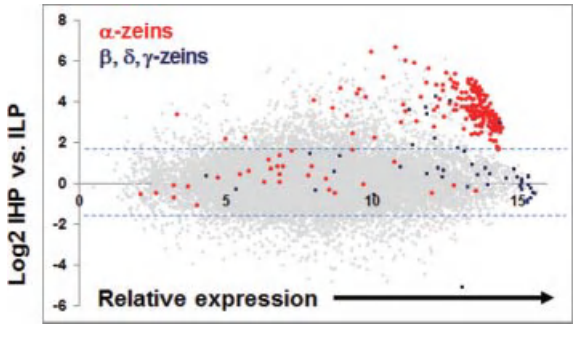

Plate 12.3 Relative zein gene expression levels in 16 DAP seeds as determined by microarray. Plotted is the expression ratio of IHP1 relative to ILP1 versus average probe signal intensity. *Dashed lines* represent cutoffs for features exhibiting statistically significant differential intensity (*t*-test, *P* value $< .05$; FDR adjusted *q* value < 0.05). The features annotated as α-zeins are plotted in red, and β-zeins, δ-zeins, and γ-zeins are plotted in blue. All other features are plotted in gray.

Plate 12.4 Phenotypes of *opaque2* introgressions into IHP1 and B73 inbred lines. *A,* Wild-type IHP1 ear. *B,* IHP1 ear homozygous for the *o2* mutation. *C,* SDS-PAGE of total seed proteins from IHP1, B73, and o2 introgressions into each of these genetic backgrounds. The relative migration of the 22-kDa α-zeins is indicated.

Plate 12.6 Photographs of kernels produced following backcrosses of *FL2-mRFP* transgenic events to the Illinois Protein Strains inbreds (TG event 52) and B73 (TG event 47). Photographs were taken at regular intervals during the period of zein accumulation from 12–24 days after pollination (DAP).

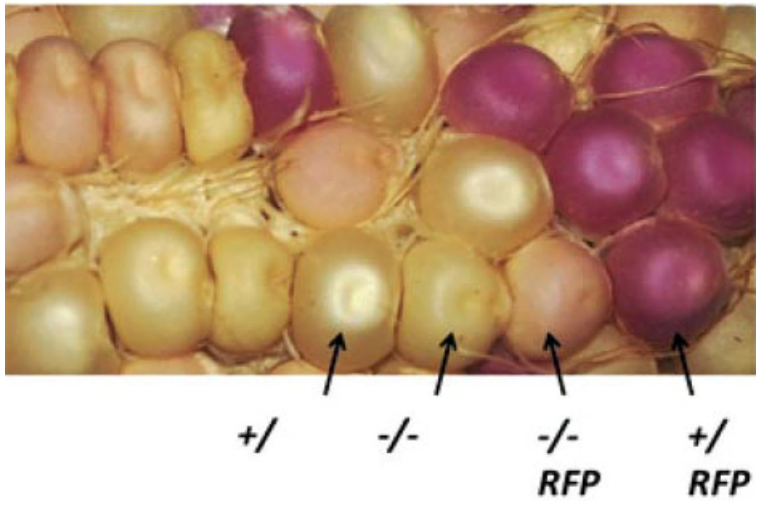

Plate 12.8 Photograph of an IHP1 ear segregating for the *o2* mutation and the *FL2-mRFP* transgene. The *o2* mutant phenotype is chalky and opaque in appearance (−/−) compared with wild-type kernels (+/). Kernels containing both the *o2* mutation and the *FL2-mRFP* transgene (−/−; RFP) illustrate reduced expression of *FL2-mRFP*, as detected by a significant decrease in pink coloration of the kernels compared with kernels containing only the *FL2-mRFP* transgene, which are much darker (+/; RFP).

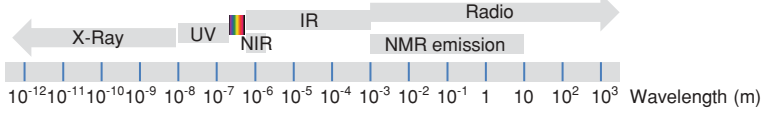

Plate 13.1 Electromagnetic spectrum showing wavelength ranges for machine vision technologies.

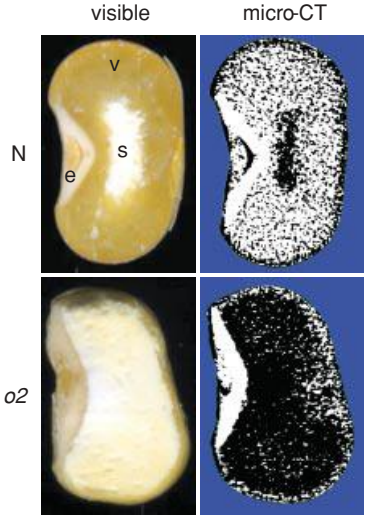

Plate 13.2 Comparison of seed cross sections from normal (N) and *opaque2* (*o2*) mutant. Visible panels are hand sections made after completing CT scans. Micro-CT scans have an identical threshold for attenuation with white pixels indicating regions above the density threshold and black pixels indicating regions below. *v,* vitreous endosperm; *s,* starchy endosperm; *e,* embryo.

Introduction

Philip W. Becraft

Agrarian civilization arose independently several times around the world. One of the earliest events occurred in the Fertile Crescent encompassing the Tigris and Euphrates river valleys of what is presently southeastern Turkey and northern Syria (Lev-Yadun *et al.*, 2000). This is believed to have occurred as early as 11,000 BP and to have involved cultivation of seven founder crops: einkorn wheat, emmer wheat, barley, lentil, pea, bitter vetch, and chickpea. At roughly the same time, early agriculture was occurring in the Yangtze valley of China centered on rice cultivation (Zhao, 2010) and in Mesoamerica involving primarily maize, beans, and squash (Zizumbo-Villarreal and Colunga-GarcíaMarín, 2010). It is notable how prevalent seeds and grains are among these early crops, and this is no accident but due to their high nutritional content and amenability to long-term storage without spoiling. With the advent of agriculture and the resultant stable food supplies came the ability to form permanent settlements, which led ultimately to the rise of modern civilization.

Amazingly, we remain as dependent as ever on seed crops. According to the Food and Agriculture Organization of the United Nations (FAO Statistical Yearbook 2012; http://www.fao.org/), an estimated 50% of global human dietary calories come directly from cereal grains. This figure represents a decline in recent decades, which is largely attributed to increased consumption of calories from vegetable oils, primarily derived from oilseed crops. Livestock products, including dairy, account for only about 13% of human calories, and much of that is indirectly derived from seed-based feeds. Thus, most human caloric intake derives from seed crops.

Seed science has never faced more important challenges or more exciting opportunities than at the present time. As human populations continue to grow, fuel costs soar, and climate change progresses, agriculture will face ever-increasing pressure to produce more food and biofuel, with lower inputs and under increasingly adverse environmental conditions. It is paramount that research investments be made to keep ahead of these growing challenges. New genomic technologies allow biological systems to be studied on scales and at depths not possible just a few years ago. These technologies are providing new insights into the fundamental biology of seed development and metabolism and leading to new strategies for improving seed traits through biotechnical approaches and breeding.

Seed biology is fascinating and complex. Seeds must survive a highly desiccated state and remain quiescent for an indeterminate period of time, then on sensing favorable environmental conditions, reactivate metabolic processes and initiate germination. Seed development involves the coordinated activities of three genetically distinct entities: the embryo, the endosperm, and the maternal plant. The embryo represents the next plant generation; the endosperm is a support tissue that nourishes

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the embryo and, in some species, the germinating seedling; and the maternal tissues contribute the protective and dispersal functions of the seed coat and pericarp. During the morphogenetic phase, the basic body plan of the embryo is established. During filling, storage products accumulate, and finally during seed maturation, tissues acquire the highly specialized ability to survive seed drying and often develop dormancy to ensure against premature germination. The accumulated storage products include starches, oils, proteins, and minerals. They are required to nourish the germinating seedling until it can become established, produce its own photosynthate, and acquire its own mineral nutrients. These storage products are also what make seeds valuable as crops.

This book contains contributions from internationally renowned scientists who describe the application of genomic analyses to various aspects of seed research and improvement. The primary focus of the book is biological rather than technical, although a wide spectrum of technical approaches and considerations are described throughout. In Chapter 1, David Meinke, one of the pioneers of large-scale seed mutant analysis in *Arabidopsis*, provides a historical perspective on the field and his group's contributions. He discusses the SeedGenes database, which compiles a vast reservoir of community information and data on existing seed mutants and the corresponding genes. This chapter illustrates one of the most important ongoing challenges in the genomics era: storing and managing huge amounts of data and presenting it in a format that is accessible and useful to the research community.

Chapters 2 and 3 provide detailed accounts of the processes of embryogenesis and endosperm development, emphasizing their genetic regulation. The embryo produces the next generation of sporophyte plant. Embryogenesis begins with a single-celled zygote and through processes of pattern formation and morphogenesis produces an embryo containing the basic body plan that is perpetuated throughout the life of the plant. The endosperm derives from a second fertilization event and serves as a support tissue to nourish the embryo during early embryogenesis. In species with persistent endosperm, such as cereals, the endosperm also nourishes the germinating seedling until it can become established. In addition to their biological significance, both structures serve as reservoirs for seed storage compounds, which are of value to humans. Both chapters highlight the complexities of these systems, illustrating the power of single-gene mutant analyses and their inherent limitations and the need for systems biology approaches that fully integrate data to understand the interacting networks that simultaneously occur at different levels (e.g., transcriptomic, proteomic, metabolomic).

Endosperm also exhibits gene imprinting, whereby maternally inherited versus paternally inherited alleles show differential expression because of epigenetic regulation. The adaptive functions and molecular mechanisms of this phenomenon are presented in Chapter 4. It appears to be involved in regulating nutrient allocation to developing seeds with implications for seed yield as well as maintaining genome integrity by suppressing transposon activity during reproduction. One exciting aspect of imprinting is that some of the molecular machinery appears to be involved in repressing seed development until triggered by fertilization, which could relate to apomixis. Apomixis is the fertilization-independent formation of seeds that retain the identical genetic constitution of the mother plant. As discussed in Chapter 5, apomixis has enormous economic potential because of the possibility of fixing hybrid vigor, and more recent progress suggests it might soon be possible to engineer apomixis into sexual crop species.

Seeds occupy a critical phase in the plant life cycle, and seed dormancy controls the timing of germination to maximize the likelihood that seedlings will be met with favorable conditions to establish, grow, and complete their reproductive cycle. The many mechanisms of dormancy allow different species to exist in their respective ecological niches by synchronizing germination to the various limiting conditions present in different environments (e.g., temperature or moisture).

Dormancy is also a critical agronomic trait; inadequate dormancy can result in crop yield losses owing to preharvest sprouting, whereas overly dormant seeds might fail to germinate when planted resulting in poor stand establishment. Chapter 6 discusses ongoing approaches to dissect the complex regulation of seed dormancy.

As mentioned, the major value of seeds to humans comes from the storage compounds they accumulate, primarily proteins, oils, and starch as well as minerals and secondary metabolites. In addition to nourishing the germinating seedling, these compounds contribute to the nutritional value of seeds for human or livestock consumption, providing energy and protein as well as other dietary benefits such as antioxidants and fiber. These compounds have found increasing use more recently in industrial applications, including biofuels, plastics, and more. Not only is the yield of these various compounds important but also the quality. The biochemical differences in seed composition impact the end use of seeds by affecting things such as baking characteristics of flour, flavor or heat tolerance of oils, or the digestibility of starch. Chapters 7–10 discuss starches, proteins, and oils, including their metabolism and factors that affect their accumulation and quality for various end uses. A common theme for all these compounds is the surprising complexity in their metabolism and how subtle structural variation can influence their physical properties. For example, starch with nothing but polymers of glucose subunits connected by α1-4 or α1-6 glycoside bonds shows dramatic differences in things such as gelling properties and digestibility, depending on the particular arrangement of the bonds and molecular packing into granules. There is a large repertoire of enzymes, not fully understood, that confer these molecular properties to the starch molecule. Proteins and oils are similarly diverse and complex. Genetic and genomic studies, including comparative genomics of different species, are lending insights to how variation in such properties are controlled and how these storage systems evolved.

In addition to the storage compounds that accumulate in seeds, another valuable seed product is cotton fiber, which is important in the textile industry and for other uses. Chapter 11 describes genomic studies in cotton where the most important seed trait is fiber. Ongoing studies seek to understand the genomic underpinnings controlling fiber quality and yield. This also serves as a model for studying processes of plant cell growth and cell wall deposition. Studies on the establishment of fiber cell fate specification provide an excellent example of translational research where basic research in *Arabidopsis* trichome development directly contributes to the understanding of an economically important trait. Cotton is also a model polyploid system for studying the negotiations and accommodations that occur between independent genomes when they are combined.

One of the most exciting areas of crop genomic science is at the interface with crop breeding. After all, the ultimate goal of plant genomics research is for crop improvement. The Illinois Long-Term Selection Project is a unique resource where a single starting maize population has been subjected to $>$110 cycles of continuous selection for seed traits including protein and oil content. These selection schemes have been reversed for several subpopulations, lines have been crossed to create mapping populations, germplasm has contributed to breeding programs, and, more recently, genomic analyses have been applied to these populations. As described in Chapter 12, this has provided new insights into genome-level responses to long-term selection, which will have bearing on one of the great questions pondered by plant breeders (or probably more often by nonbreeders): "When will the genetic variation run out?"

Finally, phenotypic analysis is often cited as the bottleneck to high-throughput studies. In closing, Chapter 13 discusses various spectral imaging technologies that are being combined with computer algorithms to develop high-throughput, automated systems for analyzing seed traits. As described, these approaches afford the opportunity to gather much more information in a single measurement than is possible with manual techniques and to do it more quickly and more accurately. Some of

these imaging techniques provide three-dimensional spatial information as well as compositional information. Furthermore, the data are preserved and can often be mined for additional information as new computer algorithms are developed. This area holds tremendous promise for future advancement as new imaging technologies are developed and applied to the analysis of seed traits. When combined with genomic studies, basic research on seed biology and breeding for improved seed traits can be greatly accelerated, and genetic potentials can be realized.

I thank the authors for their outstanding contributions. Their efforts make readily accessible an enormous amount of information, some of which was previously unpublished. I greatly enjoyed working on this project and found each of the chapters exciting and educational. I hope you find it valuable, too.

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1 Large-Scale Mutant Analysis of Seed Development in *Arabidopsis*

David W. Meinke

Introduction

With advances in DNA sequencing and reports of sequenced genomes appearing at an accelerating rate, one can easily forget an important principle that first guided research in molecular plant biology 25 years ago – that genomics and proteomics are most powerful when focused on model genetic organisms. It is therefore fitting that a book devoted to seed genomics should include several chapters on the use of genetic analysis to address fundamental questions in seed biology. My objective in this chapter is not to detail all of the seed mutants analyzed to date or to describe all of the biological questions that have been addressed with these mutants. Instead, I have chosen to focus on my own professional journey, spanning the past 35 years, to isolate and characterize large numbers of embryo-defective (*emb*) mutants in the model plant, *Arabidopsis thaliana*. This choice is justified by a quick look at the numbers involved. More embryo mutants have been isolated and characterized in *Arabidopsis*, and their genes identified, than in all other angiosperms combined. Any discussion of the strategies, procedures, and conclusions drawn from the analysis of large numbers of mutants defective in seed development must therefore focus on what has been accomplished in *Arabidopsis*. This work has been performed over several decades by dozens of individuals in my laboratory, along with scores of investigators throughout the *Arabidopsis* community. The results summarized in this chapter are a testament to their combined efforts and insights. Readers unfamiliar with basic features of seed development are referred to Chapters 2 and 3 of this book.

Historical Perspective

Mutants defective in seed development have long played an important role in genetic analysis (Meinke, 1986, 1995) – from Mendel's wrinkled seed phenotype in pea, which results from transposon inactivation of a starch-branching enzyme (Bhattacharyya *et al.*, 1990), to studies by early plant geneticists on germless (embryo-specific) and defective kernel (*dek*) mutants of maize (Demerec, 1923; Mangelsdorf, 1923; Emerson, 1932) and the nature of embryo-endosperm interactions during seed development (Brink and Cooper, 1947). Large-scale mutant analysis of seed development in maize began in the late 1970s with the isolation and characterization of several hundred *dek* mutants generated following ethyl methanesulfonate (EMS) pollen mutagenesis (Neuffer and

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Sheridan, 1980; Sheridan and Neuffer, 1980). Another 64 *dek* mutants, along with 51 embryo-specific mutants, were later described in genetic stocks known to contain the transposable element, Robertson's Mutator (Clark and Sheridan, 1991; Sheridan and Clark, 1993; Scanlon *et al.*, 1994). Although many additional mutants of this type have likely been encountered in screens of other transposon insertion lines, a global analysis of all disrupted genes associated with kernel phenotypes in maize has not been published. Attention has focused instead on a detailed characterization of selected mutants of particular interest (José-Estanyol *et al.*, 2009), including the *dek1* mutant defective in aleurone cell identity (Becraft *et al.*, 2002, Tian *et al.*, 2007; Yi *et al.*, 2011) and a number of viviparous mutants that exhibit premature germination (Suzuki *et al.*, 2003, 2006, 2008). By contrast, seed mutants in other grasses such as rice (Hong *et al.*, 1996; Kamiya *et al.*, 2003; Kurata *et al.*, 2005) have been examined in much less detail, with most genetic studies focused on other phenotypes of interest.

The isolation and characterization of embryo-lethal mutants of *Arabidopsis* was first described by Andreas Müller in Gatersleben, Germany. Müller (1963) characterized 60 mutants with different embryo phenotypes, including defects in embryo pigmentation, demonstrated that mutant and wild-type seeds could be distinguished in heterozygous siliques, and established the "Müller embryo test" to assess the mutagenic effects of ionizing radiation and chemical treatments in *Arabidopsis*. Although his attention was later directed to other systems, Müller remained particularly interested in fusca mutants, which accumulate anthocyanin during embryo maturation (Miséra *et al.*, 1994). Original stocks of the other mutants identified by Müller (1963) were not maintained.

I started to work on *Arabidopsis* as a graduate student in the laboratory of Ian Sussex at Yale University. My Ph.D. dissertation described the isolation and characterization of six embryo-lethal mutants of *Arabidopsis* and the value of such mutants in the study of plant embryo development (Meinke and Sussex, 1979a, 1979b). This work began at a time when *Arabidopsis* was known more for research in biochemical genetics than in developmental or molecular genetics. After completing a postdoctoral project on soybean seed storage proteins with Roger Beachy at Washington University in St. Louis (Meinke *et al.*, 1981), I moved to Oklahoma State University, where I focused my attention again on embryo mutants of *Arabidopsis*. My initial strategy was to analyze additional mutants isolated following EMS seed mutagenesis (Meinke, 1985; Baus *et al.*, 1986; Heath *et al.*, 1986; Franzmann *et al.*, 1989). Because some mutant seeds were capable of germinating and producing defective seedlings in culture, I adopted the term "embryo defective" (*emb*) rather than "embryo lethal" to describe the expanding collection. This nomenclature has been used ever since, although some *EMB* locus numbers were later replaced with more informative symbols (*sus*, *twn*, *lec*, *bio*, *ttn*) to indicate phenotypes of special interest.

A different approach to genetic analysis of plant embryo development was first described 20 years ago in a publication from Gerd Jürgens' laboratory in Germany (Mayer *et al.*, 1991). Rather than attempt to analyze every mutant defective in embryo development, the Jürgens group focused attention on a small number of mutants with defective seedlings that appeared to result from alterations in embryo pattern formation. As described elsewhere in this book, several of these mutants uncovered important cellular pathways associated with plant embryo development, although in many cases, the gene products were unexpected and did not appear to support the original hypothesis, based on work with *Drosophila*, that embryo patterning mutants should identify transcription factors that regulate developmental decisions. Whereas my approach was to "cast a wide net" and explore interesting stories based on the analysis of many different types of mutants, the Jürgens group focused on a limited set of phenotypes defined by a handful of genes with multiple alleles and identified gene networks associated with those phenotypes. In retrospect, both of these approaches were required to develop a comprehensive picture of the genetic control of plant embryo development.

Table 1.1 Experimental Features That Make *Arabidopsis thaliana* an Attractive System for Large-Scale Mutant Analysis of Seed Development

Arabidopsis Feature	Relevance of Feature to Genetic Analysis of Seed Development
Self-pollinated flowers	Crosses not required to maintain *emb* mutants and most genetic stocks
Indeterminate inflorescences	Mature plants contain large numbers of siliques at different stages of development, arranged in a predictable progression along each stem; facilitates identification of embryos at desired stage of development
Transparent seed coat	Wild-type seeds at the cotyledon stage are green and can be readily distinguished from unfertilized ovules and aborted seeds
Spontaneous seed abortion rare	Facilitates identification of mutant seeds in heterozygous siliques
Small seed size at maturity	Embryos within immature seeds are readily observed with Nomarski (DIC) light microscopy; optical sectioning through immature seeds possible
Siliques contain 50–60 seeds	Segregation of normal and mutant seeds readily observed in 1 silique
Short pollen-tube growth path	Facilitates recovery of mutants defective in both embryo and gametophyte development

Arabidopsis Embryo Mutant System

The advantages of *Arabidopsis* as a model system for research in plant biology are well known (Redéi, 1975; Meyerowitz and Somerville, 1994; Meinke *et al.*, 1998; Koornneef and Meinke, 2010). Important features that make *Arabidopsis* suitable for large-scale mutant analysis of seed development have also been described (Meinke, 1994). Several of these features are highlighted in Table 1.1. Recessive embryo-defective mutants are maintained as heterozygotes, which typically produce 25% mutant seeds after self-pollination. Because each silique contains 50–60 total seeds and multiple siliques are arranged in a developmental progression along the length of each stem, mutant seeds at many different stages of development can be found on a single plant at maturity. Mutant and normal seeds can be readily distinguished, based on size, color, and embryo morphology, by screening immature siliques under a dissecting microscope. Mutant embryos that have reached an advanced globular stage can be removed with fine-tipped forceps and examined further; embryos arrested at earlier stages of development are best observed under a compound microscope equipped with Nomarski (differential interference contrast [DIC]) optics. After seed mutagenesis, siliques of chimeric M_1 plants can be screened to identify flowers that arose from the mutant sector (Meinke and Sussex, 1979a, 1979b). Mature siliques derived from this sector are harvested to collect dry seeds. After germination, heterozygous and wild-type plants often segregate in a 2:1 ratio. If insertion lines are involved and the disrupted *EMB* gene is associated with a selectable marker, the appropriate selection agent can be used to identify heterozygous plants at the seedling stage, provided that there are no additional inserts located elsewhere in the genome. With EMS mutants, heterozygous plants cannot be distinguished from wild-type plants until selfed siliques have matured and are screened for defective seeds. When plants segregating for an *emb* mutation are crossed for allelism tests, parental heterozygotes must be identified before the cross can be performed, which limits the time available for crosses to be completed. Allelic mutants that fail to complement result in siliques with 25% mutant seeds; mutants disrupted in different genes typically produce siliques with all normal seeds.

Large-Scale Forward Genetic Screens for Seed Mutants

In contrast to screens for most visible phenotypes in *Arabidopsis*, which involve the identification of homozygotes in a second (M_2) generation following seed mutagenesis, forward genetic screens for embryo-defective mutations can be performed directly on M_1 plants. This approach was used

to isolate most of the original *emb* mutants analyzed in my laboratory (Meinke, 1985). When Ken Feldmann developed a method for *Agrobacterium*-mediated seed transformation in *Arabidopsis* and began to grow large populations of T-DNA insertion lines at DuPont in the late 1980s (Feldmann, 1991), two different groups were involved in screening the populations for embryo-defective mutations. One group, comprising investigators associated with Robert Goldberg's laboratory at UCLA (Yadegari *et al.*, 1994), screened half of the plants; members of my laboratory screened the other half (Errampalli *et al.*, 1991; Castle *et al.*, 1993). The same strategy was used with a second population of plants that Feldmann made available several years later at the University of Arizona. My approach to the analysis of these populations was first to determine which mutants were tagged with T-DNA and which were not tagged. About two thirds of the lines that segregated for an embryo-defective mutation were not amenable to rapid gene identification because they fell into the second category. The method used to resolve tagging status involved transplanting kanamycin-resistant seedlings derived from selfed heterozygotes to soil and determining whether all of those plants produced siliques with 25% mutant seeds, as expected if a single T-DNA insert was present and disrupted an *EMB* gene. When additional inserts were involved, we identified subfamilies in future generations that contained a single insert and then proceeded with the analysis described above. For mutants examined in my laboratory, the original *emb1* to *emb69* alleles were identified after EMS (or in some cases x-ray) seed mutagenesis, *emb71* to *emb180* mutants involved the DuPont collection, and the *emb200* series was reserved for the Arizona collection. Most of these *EMB* loci are listed in Meinke (1994) and in the "Archival Data on Meinke Lab Mutants" link at the SeedGenes website devoted to genes with essential functions during seed development in *Arabidopsis* (www.seedgenes.org). In some cases, the gene responsible for the mutant phenotype has since been identified. In many cases, however, the association between mutant phenotype and gene function remains to be determined.

A major breakthrough in forward genetic analysis of seed development occurred in the late 1990s, when David Patton and Eric Ward at Ciba-Geigy, which later became Syngenta (Research Triangle Park, NC), embarked on a large-scale, forward genetic screen for essential genes of *Arabidopsis*. The rationale was that some essential gene products identified through such efforts might represent promising targets for novel herbicides. Over the next 15 years, in close collaboration with my laboratory, >120,000 T-DNA insertion lines were screened for seed phenotypes, including embryo and seed pigment defects, >1600 promising mutants were isolated and characterized, ~440 tagged mutants were identified, and ~200 gene identities were revealed (McElver *et al.*, 2001). Of equal importance, Syngenta ultimately agreed to make most of these gene identities public, after they had been evaluated in house (Tzafrir *et al.*, 2004). This provided the foundation for a large-scale NSF 2010 project in my laboratory that established, in collaboration with Allan Dickerman at the Virginia Bioinformatics Institute, a comprehensive database of all known essential genes required for seed development in *Arabidopsis* (Tzafrir *et al.*, 2003). Results of the "SeedGenes" project are described later in this chapter.

Approaches to Mutant Analysis

The belief that lethal mutants are not useful or informative because they cannot be analyzed in detail is misguided. Sometimes the terminal phenotype alone is sufficient to offer valuable insights. Abnormal suspensor (*sus*) and twin (*twn*) mutants provided early support for the idea that the embryo proper normally restricts the developmental potential of the suspensor (Marsden and Meinke, 1985; Schwartz *et al.*, 1994; Vernon and Meinke, 1994). The leafy cotyledon (*lec*) mutant phenotype (Meinke, 1992; Meinke *et al.*, 1994) indicated that the default state for cotyledons is a leaflike

structure, consistent with the evolution of cotyledons as modified leaves. The initial steps in mutant analysis often involve determination of segregation ratios of mutant seeds in heterozygous siliques and the use of dissecting microscopes and light microscopy to characterize the terminal embryo phenotype. A reduced frequency or unusual distribution of mutant seeds in heterozygous siliques frequently indicates an additional role of the disrupted gene in male gametophyte development (Meinke, 1982; Muralla *et al.*, 2011). A mixture of aborted seeds and unfertilized ovules often indicates a role in female gametophyte development (Berg *et al.*, 2005). Examination of mutant seeds with Nomarski optics or sectioned material with light or electron microscopy can reveal unexpected features, such as incomplete cell walls in the *cyt1* mutant (Nickle and Meinke, 1998) and giant endosperm nuclei and enlarged embryo cells in titan (*ttn*) mutants (Liu and Meinke, 1998; Liu *et al.*, 2002; Tzafrir *et al.*, 2002). Light microscopy, in combination with gel electrophoresis of seed proteins, can also be used to determine whether mutant embryos unable to complete morphogenesis continue to differentiate at the cellular level (Heath *et al.*, 1986; Patton and Meinke, 1990; Yadegari *et al.*, 1994).

The original idea behind examining the response of mutant embryos in culture was to determine whether mutant seedlings or callus tissue could be produced for further analysis and to search for auxotrophic mutants that survived on enriched media containing the required nutrient (Baus *et al.*, 1986). This approach resulted in the successful identification of the first auxotrophic mutant (*bio1*) known to be associated with embryo lethality in *Arabidopsis*, and it helped to explain the scarcity of plant auxotrophs identified at the seedling stage (Schneider *et al.*, 1989). Another mutant (*bio2*) was later found to be blocked at a different step in the same pathway (Patton *et al.*, 1998). The chromosomal deletion associated with this mutant allele also contributed, by chance, to the identification of a closely linked gene (*FPA*) involved in floral induction (Schomburg *et al.*, 2001). Reverse genetic analysis later revealed that *BIO1* is part of a complex (*BIO3–BIO1*) locus that encodes a fusion protein responsible for two sequential steps in biotin synthesis (Muralla *et al.*, 2008). Embryo culture experiments also enabled further characterization of mutants with late defects in embryo development (Vernon and Meinke, 1995) and resulted in the identification of two mutants with especially striking phenotypes: *lec1* (Meinke, 1992) and *twn1* (Vernon and Meinke, 1994).

Another approach to mutant analysis that can occur independently of gene isolation involves mapping the chromosomal locations of *EMB* genes relative to morphological or molecular markers. We used this approach to enhance the classical genetic map of *Arabidopsis* and to facilitate the identification of potential mutant alleles suitable for genetic complementation tests (Patton *et al.*, 1991; Franzmann *et al.*, 1995). More recently, we performed allelism tests between mapped (but not cloned) mutants and cloned (but not mapped) mutants to identify new alleles of cloned *EMB* genes and reveal the identities of mapped *EMB* genes (Meinke *et al.*, 2009b). Classical genetic mapping with *emb* mutants is enhanced by the fact that heterozygous plants can be identified by screening selfed siliques for the presence of defective seeds. After completion of the genome sequence, the classical genetic map was found to have many regions inconsistent with the known order of genes along the chromosome. This finding led to the establishment of a sequence-based map of genes with mutant phenotypes to document the confirmed locations of genes previously found on the classical genetic map (Meinke *et al.*, 2003). An expanded version of this dataset, which includes 2400 *Arabidopsis* genes with a loss-of-function mutant phenotype, was recently published (Lloyd and Meinke, 2012).

Because of their small size, *Arabidopsis* seeds were once viewed as inaccessible to biochemical or molecular analysis. With continued technological advances and the development of alternative methods for detecting trace substances of interest, some of these barriers have been removed. One example from my laboratory involved the use of sensitive microbiological assays to demonstrate that

arrested embryos from the *bio1* mutant of *Arabidopsis* contain reduced levels of biotin (Shellhammer and Meinke, 1990). A more recent example is the demonstration that arrested embryos from the *sus1* mutant contain altered profiles of miRNAs, consistent with the known function of the *SUS1/DCL1* gene (Schauer *et al.*, 2002) in promoting the formation of miRNAs (Nodine and Bartel, 2010). Although seed size continues to be an issue for some *Arabidopsis* experiments, all plant embryos begin as single cells, which means that analyzing trace materials during early embryo development will continue to present unique challenges, regardless of the final size of the embryo at maturity.

Ultimately, the most powerful approach to the large-scale analysis of mutants defective in seed development involves identifying the disrupted genes. Although some *EMB* genes have been identified through map-based cloning, most were identified by amplifying genomic sequences flanking insertion sites in T-DNA tagged mutants. Overall, 80% of the *emb* mutants found in the SeedGenes database were generated with T-DNA insertional mutagenesis compared with 9% with transposable elements and 9% with EMS. Advances in polymerase chain reaction (PCR)–based strategies for insertion site recovery played a critical role in identifying large numbers of genes required for seed development in *Arabidopsis*. For *EMB* genes analyzed in my laboratory, *EMB* numbers 1000 through 2750 denote genes uncovered through forward genetic screens of Syngenta insertion lines; *EMB* numbers 2761 through 2820 indicate genes uncovered through reverse genetic screens, often involving Salk insertion lines; *EMB* numbers 3002 to 3013 correspond to French insertion lines (Devic, 2008); and *EMB* numbers 3101 to 3147 correspond to lines first tested in the laboratory of Kazuo Shinozaki at the Riken Plant Science Center in Japan (Bryant *et al.*, 2011).

Strategies for Approaching Saturation

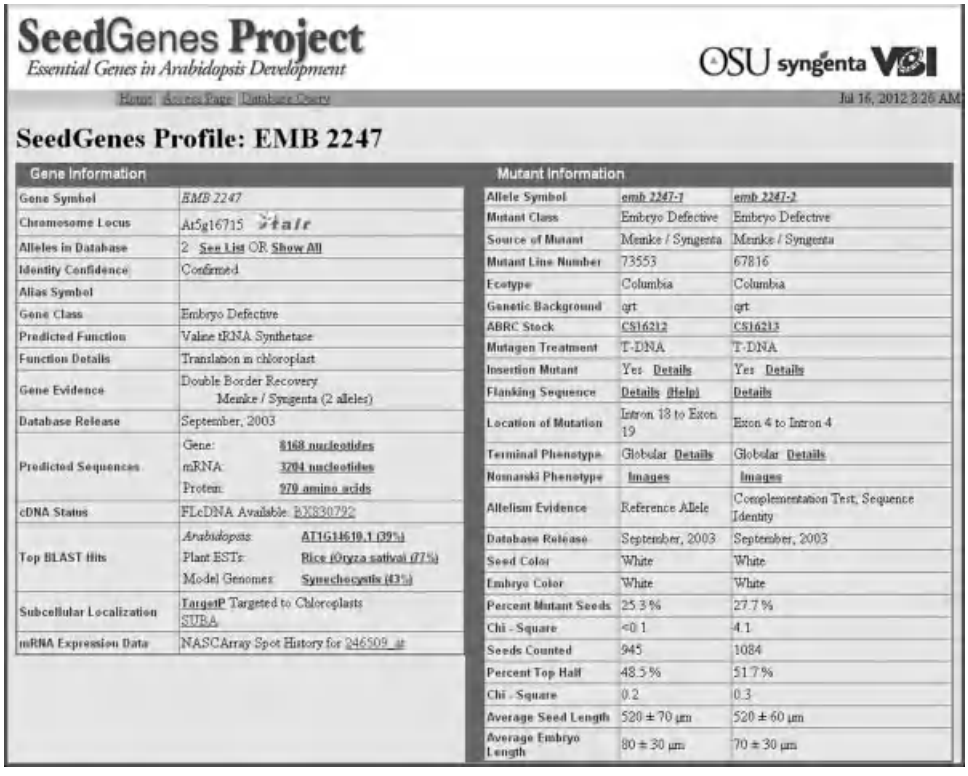
Forward genetics eventually becomes an inefficient strategy for identifying *EMB* genes because many of the new mutants examined represent alleles of known *EMB* genes. This trend has already been observed in *Arabidopsis*, with duplicate mutant alleles frequently encountered in mapped populations (Franzmann *et al.*, 1995; Meinke *et al.*, 2009b) and sequenced insertion lines (McElver *et al.*, 2001; Tzafrir *et al.*, 2004). A substantial number of mutants analyzed in detail in other laboratories have also turned out to be allelic to mutants first identified in my laboratory. About 8 years ago, we began to explore reverse genetic strategies for approaching saturation by focusing on *EMB* gene candidates not found through forward genetics. Promising candidates included *Arabidopsis* orthologs of known essential genes in other model organisms (Tzafrir *et al.*, 2004); genes encoding proteins that function in a shared biosynthetic pathway (Muralla *et al.*, 2007, 2008), cellular process (Berg *et al.*, 2005), or intracellular compartment (Bryant *et al.*, 2011) as a known EMB protein; and genes encoding a protein interactor of a known *EMB* gene product. We also analyzed hundreds of insertion lines that appeared from other studies (O'Malley and Ecker, 2010) to lack insertion homozygotes (Meinke *et al.*, 2008), which we reasoned might indicate embryo or gametophyte lethality. Although dealing with insertion lines on a large scale can be problematic, dozens of additional *EMB* genes were identified through a combination of these approaches. Reverse genetics was also used to find second alleles of genes first identified through forward genetics. When accompanied by genetic complementation tests, these additional alleles confirmed that the gene responsible for the mutant phenotype had been identified. The most difficult problem with Salk insertion lines (Alonso *et al.*, 2003) was reduced expression of the kanamycin-resistance marker, which meant that efficient methods developed for demonstrating close linkage between the disrupted gene and mutant phenotype based on selection, transplantation, and screening protocols (McElver *et al.*, 2001) were replaced by PCR genotyping, which is more expensive and subject to errors. In a substantial number of cases,

the predicted insert could not be found or did not cosegregate with the phenotype. Similar problems with large-scale screens of Salk insertion lines have been described elsewhere (Ajjawi *et al.*, 2010). Populations of insertion lines with a more consistent selectable marker, including the GABI (Rosso *et al.*, 2003) and Riken (Kuromori *et al.*, 2004) collections, were more efficiently analyzed, but unexplained results were still encountered, and decisions had to be made about whether to resolve the ambiguities or move ahead with additional candidates. Some *EMB* candidates confirmed with reverse genetics also turned out to be the subject of ongoing studies in other laboratories, which meant that unwanted duplication of effort was involved. Because of these added complications, we eventually abandoned reverse genetic analysis on a large scale and began to focus instead on further analysis of the existing collection of *EMB* genes.

SeedGenes Database of Essential Genes in *Arabidopsis*

One goal of my NSF 2010 project was to establish a public database that summarized information on genes required for seed development in *Arabidopsis*. The resulting database (www.seedgenes.org) was first released in 2002 and has since been updated multiple times. Allan Dickerman at the Virginia Bioinformatics Institute assisted with construction of the database and oversees its maintenance. The most recent (eighth) database release (December 2010) includes information on 481 genes and 888 mutants. Over 60% of the mutants have been analyzed in my laboratory. Information about the remaining mutants was extracted from the literature. Three classes of mutants are included in the database: embryo defectives, mutants with a pigment-defective embryo (albino, pale green, fusca) of normal morphology, and mutants that produce 50% rather than 25% defective seeds after self-pollination. On entering the database, users encounter the "Access Page," which provides links to lists of genes and mutants found in the database, supplemental and archival datasets, additional information on mutant collections, a tutorial on analyzing embryo-defective mutants, and details on project objectives and participants. The linked "Query Page" is divided into two different parts: gene information and mutant information. Users can browse a list of all genes or mutants, determine which genes of interest are included in the database, and generate lists of genes or mutants that match desired criteria. Database terms are linked to a glossary that provides further details. Each gene is associated with a "Profile" page, which summarizes relevant gene information on the left side of the page and mutant information on the right side. Figure 1.1 shows an example of a Profile page. From this page, users can link to further details on insertion sites for Syngenta mutants, phenotype details and Nomarski images for mutants analyzed in my laboratory, relevant sequence information, and top BLAST hits. Dividing the database into distinct but connected sections for gene and mutant information was critical for data management and represents a key design feature that could be used to develop similar databases for other model plants.

Deciding how to present phenotype information in the database was a major challenge, in part because the project evolved over a period of years and involved multiple student assistants with varying degrees of expertise. For Syngenta lines and mutants that my laboratory analyzed in some detail, we established a standardized set of terminal phenotypes (Figure 1.2) based on embryo morphology as visualized under a dissecting microscope. We also captured Nomarski images of mutant embryos inside the developing seed (Figure 1.3). Details of these methods are given at the tutorial section of the SeedGenes website. Although this approach provided insights into the stage of developmental arrest and the diversity of embryo phenotypes observed, subtle differences in cell division patterns and defects that first distinguished mutant from wild-type embryos were generally not recorded. The SeedGenes database should therefore be viewed as a broad community resource



SeedGenes Project
Essential Genes in Arabidopsis Development
OSU syngenta VBI
Home | Source Page | Database Query
Jul 16, 2012 3:26 AM

SeedGenes Profile: EMB 2247

Gene Information	
Gene Symbol	*EMB 2247*
Chromosome Locus	At5g16715 *stale*
Alleles in Database	2 See List OR Show All
Identity Confidence	Confirmed
Alias Symbol	
Gene Class	Embryo Defective
Predicted Function	Valine tRNA Synthetase
Function Details	Translation in chloroplast
Gene Evidence	Double Border Recovery Meinke / Syngenta (2 alleles)
Database Release	September, 2003
Predicted Sequences	Gene: 8168 nucleotides mRNA: 3204 nucleotides Protein: 979 amino acids
cDNA Status	FLcDNA Available: BX830792
Top BLAST Hits	*Arabidopsis*: AT1G14610.1 (39%) Plant ESTs: Rice (Oryza sativa) (77%) Model Genomes: Synechocystis (43%)
Subcellular Localization	TargetP Targeted to Chloroplasts SUBA
mRNA Expression Data	NASCArray Spot History for 246509_at

Mutant Information		
Allele Symbol	emb 2247-1	emb 2247-2
Mutant Class	Embryo Defective	Embryo Defective
Source of Mutant	Meinke / Syngenta	Meinke / Syngenta
Mutant Line Number	73553	67816
Ecotype	Columbia	Columbia
Genetic Background	qrt	qrt
ABRC Stock	CS16212	CS16213
Mutagen Treatment	T-DNA	T-DNA
Insertion Mutant	Yes Details	Yes Details
Flanking Sequence	Details (Help)	Details
Location of Mutation	Intron 18 to Exon 19	Exon 4 to Intron 4
Terminal Phenotype	Globular Details	Globular Details
Nomarski Phenotype	Images	Images
Allelism Evidence	Reference Allele	Complementation Test; Sequence Identity
Database Release	September, 2003	September, 2003
Seed Color	White	White
Embryo Color	White	White
Percent Mutant Seeds	25.3 %	27.7 %
Chi - Square	<0.1	4.1
Seeds Counted	945	1084
Percent Top Half	48.5 %	51.7 %
Chi - Square	0.2	0.3
Average Seed Length	520 ± 70 µm	520 ± 60 µm
Average Embryo Length	80 ± 30 µm	70 ± 30 µm

Figure 1.1 Screen capture of the Profile page for a representative gene (*EMB2247*) included in the SeedGenes database of essential genes in *Arabidopsis* (www.seedgenes.org). In this case, both mutant alleles were identified through a forward genetic screen of T-DNA insertion lines generated at Syngenta. Underlined and colored terms shown here link to other pages, primarily within the SeedGenes database.

Figure 1.2 Classification system for terminal phenotypes of mutant embryos removed from seeds before desiccation. For most of the Syngenta mutants analyzed in the early stages of our NSF 2010 project, we dissected 100 mutant seeds from siliques that contained normal seeds at a mature green stage of development. We then attempted to place each mutant embryo into one of the phenotype classes shown here. For stage "X," no mutant embryo was found on dissection; this usually meant that the mutant embryo was arrested before a late globular stage. Results of these phenotype screens are found by clicking on the "Details" link in the "Terminal Phenotype" section of the SeedGenes Profile page.

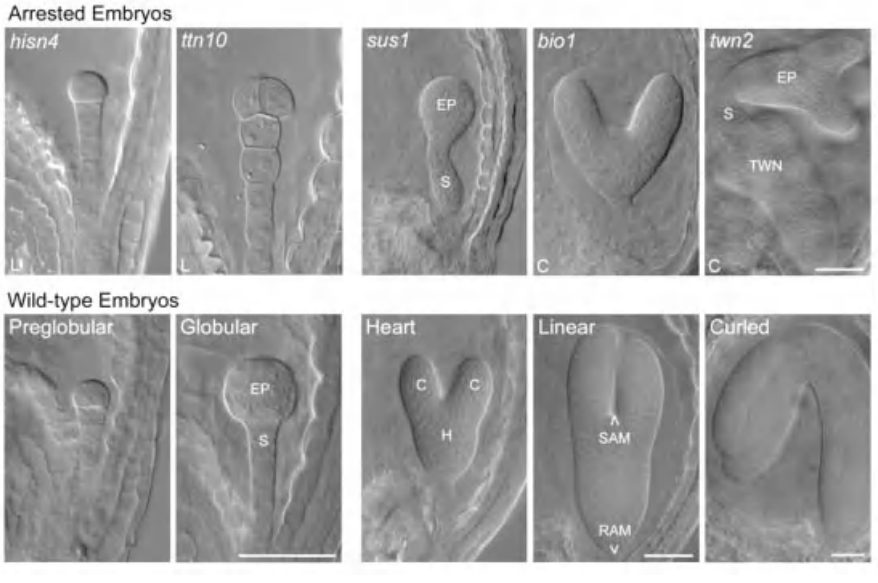


Figure 1.3 Representative collection of embryo-defective phenotypes found in the SeedGenes database. Regions of wild-type embryos include the embryo proper (EP), suspensor (S), cotyledons (C), hypocotyl (H), shoot apical meristem (SAM), and root apical meristem (RAM). Examples of aberrant development include irregular patterns of cell division, altered embryo morphology, giant suspensors, and twin embryos. The second (TWN) embryo in *twn2* arises from the suspensor (S) of the first embryo (EP). Seeds were removed from immature siliques and visualized with Nomarski (DIC) light microscopy. Arrested (mutant) embryos were obtained from heterozygous siliques at the linear (L) or curled cotyledon (C) stage of seed development. The four images on the left side are more highly magnified than the images on the right. Scale bars, 50 μm. (Modified from Meinke *et al.* [2008].)

and a starting point for additional studies rather than a definitive source of detailed phenotype information on individual mutants of interest.

Embryo Mutants with Gametophyte Defects

The more we began to characterize mutants defective in embryo development, the more important it became to distinguish between embryo and gametophyte mutants. Some gametophyte mutants of *Arabidopsis* are leaky, resulting in embryo lethality whenever fertilization takes place. In addition, some embryo mutants exhibit reduced transmission of the mutant allele and noticeable defects in gametophyte development. This raises a fundamental question: How can mutant (*emb*) gametophytes survive if an essential function required throughout the life cycle is disrupted? In other words, why do some essential gene disruptions in *Arabidopsis* result in gametophyte lethality, whereas others lead to embryo lethality?

To address this question, we first had to establish a comprehensive dataset of gametophyte essential genes in *Arabidopsis* that could be compared with the embryo dataset presented at SeedGenes. My laboratory recently created such a dataset, further edited the SeedGenes collection of *EMB* genes, and established several different categories of embryo and gametophyte mutants to facilitate comparative studies (Muralla *et al.*, 2011). The edited *EMB* dataset, which excluded six problematic SeedGenes loci, provides detailed information, including terminal phenotype classes, for 396 *EMB* genes in *Arabidopsis*. This dataset includes 352 "true *EMB*" genes required for seed development but without

a known gametophyte defect and 44 genes assigned to the EMG (Embryo-Gametophyte) subclass of embryo and gametophyte loci, which produce at least 10% defective seeds following self-pollination of heterozygotes and have a reduced frequency of mutant seeds overall, too few mutant seeds at the base of the silique, or an excessive number of aborted ovules, all of which indicate a secondary role in male or female gametophyte function. Genes assigned to the GEM (Gametophyte-Embryo) subclass of gametophyte mutants have a more significant defect in gametophyte function, with heterozygotes known or predicted to produce 2%–10% mutant seeds. The GAM (Gametophyte) subclass of mutants is characterized by even more severe defects in gametophyte function, with $<$2% mutant seeds expected from selfed heterozygotes. Other gametophyte mutants have more variable or less well-defined defects or give rise to viable homozygotes.

To examine the functional differences among these mutant classes, we compared 70 GAM genes with reduced transmission efficiency, 352 true *EMB* loci, and 72 EMG and GEM genes with defects in both embryo and gametophyte development (Muralla *et al.*, 2011). The difference between embryo and gametophyte mutants could not be explained based on protein function alone, although distinctive features of each dataset were identified. Two alternative explanations for how mutants defective in embryo development might survive gametophyte development were also discounted because neither genetic redundancy nor residual protein function in weak mutant alleles appeared to explain the different phenotypes observed. Instead, we proposed that residual gene products derived from transcription in heterozygous microsporocytes and megasporocytes often enable mutant gametophytes to survive the loss of an essential gene product and participate in fertilization, after which time the gene disruption eventually limits embryo growth and development (Muralla *et al.*, 2011).

General Features of *EMB* Genes in *Arabidopsis*

The first question about *EMB* genes that needs to be addressed concerns how many such loci are present in the genome. Our best estimate, based on the frequency of seed mutants and duplicate mutant alleles uncovered in mutagenesis experiments, is 750–1000 genes required for seed development in *Arabidopsis* (Meinke *et al.*, 2009b), which corresponds to about 3% of all protein-coding sequences. The current collection of 400 *EMB* genes likely represents at least 40% saturation, sufficient to begin evaluating salient features. Most *EMB* genes are not embryo-specific in their pattern of expression. Embryo development is simply the stage of development when the loss of gene product first becomes critical. Consistent with this idea, weak alleles of many *EMB* genes exhibit phenotypes later in plant development (Muralla *et al.*, 2011). *EMB* genes are widely distributed throughout the five chromosomes and are more likely than the genome as a whole to be present in a single copy. When functionally redundant genes encode a protein required for embryo development, the mutant phenotype is observed only in double or multiple mutants. Examples of such double mutant phenotypes have increased in recent years, reflecting greater emphasis on the use of reverse genetics to study essential cellular functions.

A second question about *EMB* genes concerns the stage of development reached by mutant embryos before seed desiccation. We recently summarized this information for 352 "true" *EMB* genes without evidence of gametophyte defects (Muralla *et al.*, 2011). This analysis updated information published before, using smaller datasets (Tzafrir *et al.*, 2004; Devic, 2008). Based on phenotype data for the strongest allele, 16% of gene disruptions cause embryo development to become arrested at a preglobular stage; 10%, at a preglobular to globular stage; 29%, at the globular stage; and 9% at the transition (heart) stage. Several examples are shown in Figure 1.3. Mutant embryos in 31%

of cases analyzed reach a more advanced, cotyledon stage of development, whereas the remaining 5% have terminal phenotypes that remain to be documented. Several factors may contribute to a delayed onset of developmental arrest in specific mutant embryos, including partially redundant genes, metabolic pathways, or cellular processes; residual protein function in weak mutant alleles; and diffusion of critical metabolites from surrounding maternal tissues. Terminal embryo phenotypes for some mutants are consistently limited to a single stage of development, whereas for other mutants, embryo phenotypes are highly variable and extend through multiple stages of development. In addition, some mutant embryos become arrested shortly after morphological defects are first detected, whereas others continue to develop and produce viable seedlings despite visible defects early in development. Several broad conclusions about mutant phenotypes can nevertheless be made: (1) *EMB* genes clearly differ in how far embryo development can proceed following their disruption; (2) a sizable number of mutants exhibit defects well before the globular stage of embryo development; (3) most of the mutant embryos arrested before the heart stage of development are white or very pale green, consistent with a disruption of chloroplast function, whereas many mutant embryos with defects at the cotyledon stage are green; (4) embryo and endosperm development are affected to a similar extent in some mutants but to different extents in other mutants; and (5) unusual phenotypes do not always result from disruption of "interesting" regulatory genes, and common phenotypes do not exclude the possibility that an important regulatory function has been disrupted.

The role of chloroplast translation in embryo development provides an informative example of how disrupting similar gene products in different plant species can result in different phenotypes. Many *EMB* genes of *Arabidopsis* encode proteins localized to plastids (Hsu *et al.*, 2010; Bryant *et al.*, 2011). Some of these are required for translation of mRNAs encoded by the chloroplast genome. Interfering with chloroplast translation results in embryo lethality in *Arabidopsis*, but in *Brassica* and maize, albino seedlings are produced instead (Zubko and Day, 1998; Asakura and Barkan, 2006). The difference appears to be related to the ability of some plant species to compensate for a disruption of fatty acid biosynthesis in chloroplasts by targeting a modified or duplicated version of the homomeric acetyl-CoA carboxylase to plastids. This homomeric enzyme can substitute for the loss of heteromeric acetyl-CoA carboxylase activity, which depends on production of one subunit (accD) encoded by the chloroplast genome (Bryant *et al.*, 2011). Overall, >20% of chloroplast-localized EMB proteins function in the biosynthesis of amino acids, vitamins, nucleotides, or fatty acids, consistent with the chloroplast localization of these pathways. Major disruptions of chloroplast function, such as interfering with protein import from the cytosol, can also result in embryo lethality, although less severe perturbations often result in reduced embryo pigmentation. By contrast, disruption of essential mitochondrial functions tends to result in gametophyte lethality (Lloyd and Meinke, 2012).

Value of Large Datasets of Essential Genes

Although some authors might argue that establishing large datasets of essential genes in plants is pointless given that all genes must perform an important function because otherwise they would not be maintained by natural selection (Pichersky, 2009), knockouts of essential genes have played a central role in the development of *Arabidopsis* as a model system and in the analysis of a wide range of important biological questions (Meinke *et al.*, 2008, 2009a). Such datasets also contribute a critical plant representative to ongoing comparative and evolutionary studies involving essential genes in microorganisms and multicellular eukaryotes (Liao and Zhang, 2008; Park *et al.*, 2008; Zhang and Lin, 2009; Chen *et al.*, 2010). Because *EMB* genes represent the most common phenotypic

marker in *Arabidopsis* and more *emb* mutants have been donated to the *Arabidopsis* stock centers (Meinke and Scholl, 2003) than any other class of mutant, efforts to identify a knockout for every *Arabidopsis* gene (O'Malley and Ecker, 2010) must address the analysis of hundreds of genes known to be required for embryo and gametophyte development.

One objective of recent work in my laboratory has been to establish a comprehensive dataset of *Arabidopsis* genes with a loss-of-function phenotype of any kind to facilitate research in functional genomics, comparative phenomics, and gene discovery relevant to agriculture, bioenergy, and the environment. Our curated dataset of 2400 genes associated with a single mutant phenotype and 400 genes that exhibit a mutant phenotype only when disrupted in combination with a redundant paralog (Lloyd and Meinke, 2012) should provide a foundation for establishing a community-based resource for evaluating genotype-to-phenotype relationships in a model plant.

In addition to enabling comparative studies with essential genes in other organisms, providing a robust collection of genetic markers for ongoing research, facilitating the analysis of essential plant processes, and highlighting *EMB* genes with unknown but essential functions that merit further study, analysis of large collections of mutants defective in embryo development has resolved important questions about the nature of plant auxotrophs; the diversity of interactions between the embryo proper, suspensor, endosperm, and surrounding maternal tissues; and the contributions of specific gene products to cell survival and reproductive development. A noteworthy example of how large datasets of essential genes can contribute to discussions of important biological questions involves the issue of when the transition from maternal to zygotic gene expression occurs during plant embryo development (Muralla *et al.*, 2011). Several reports have claimed that plant embryos are relatively quiescent until the globular stage of development and that in accordance with many animal systems, early plant embryos rely primarily on stored maternal transcripts (Vielle-Calzada *et al.*, 2000; Pillot *et al.*, 2010; Autran *et al.*, 2011). However, this model has been difficult to reconcile with the existence of *emb* mutants with preglobular defects and a zygotic pattern of inheritance. A recent report (Nodine and Bartel, 2012) seems to resolve this conflict by documenting that for most genes, maternal and paternal genomes contribute equally to transcripts found in early embryos, consistent with zygotic gene expression. This study included the analysis of reciprocal crosses and differed from previous studies in that embryos were washed vigorously to remove contaminating RNA derived from the maternal seed coat. When these results are evaluated in the context of long-standing research on embryo-defective mutants – most notably, the identification of 70 *EMB* genes with arrested embryos that fail to reach a globular stage of development (Muralla *et al.*, 2011) – there is compelling evidence for early activation of the zygotic genome in *Arabidopsis* and for the requirement of zygotic gene expression to support cellular functions after fertilization.

Directions for Future Research

Research on large-scale mutant analysis of seed development in *Arabidopsis* has reached a critical stage. Despite remarkable progress in the isolation and characterization of embryo-defective mutants and the use of these mutants to address important topics in plant biology, questions remain about the availability of funding to saturate for this class of mutants and the priority that should be given to identifying additional genes with mutant phenotypes in *Arabidopsis*. For some plant biologists, the time has come to focus instead on applying what has already been learned with *Arabidopsis* to other plant systems. Although this has long been the goal of research on model organisms, there is still much to be learned about how seed development is enabled through the coordinated expression of hundreds of genes with a variety of cellular functions. My hope is that by expanding

and coordinating efforts worldwide to characterize essential genes found through reverse genetics with *Arabidopsis*, progress will continue to be made toward reaching the goals that I first set forth in my Ph.D. dissertation – to explore large-scale genetic approaches to seed development in a model plant system and to understand how specific genes, proteins, and cellular processes regulate and support the formation of a mature embryo that contains root and shoot apical meristems, survives desiccation, and germinates to produce a viable plant.

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2 Embryogenesis in *Arabidopsis*: Signaling, Genes, and the Control of Identity

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Introduction

The embryo of *Arabidopsis thaliana* has become an important reference system in which to study key aspects of plant development. Biologically, the embryo represents the phase of the plant life cycle during which key features of the structural organization of the plant body are established – apical-basal polarity and radial symmetry. In contrast to the animal embryo, the plant embryo is not a miniature adult. It does not contain many organs found in the mature plant. In *Arabidopsis*, the embryo is especially simple, with only a root, cotyledons, and hypocotyl present. Embryos of other species, such as *Zea mays,* have some leaf development in advance of germination, but the *Arabidopsis* embryo is characterized by the superimposition of two developmental axes, the apical-basal and the radial. An unusual feature is that the pattern of cell divisions in the *Arabidopsis* embryo is highly stereotyped and predictable, and this allows the ready identification of genetic mutations that perturb cell patterning mechanisms (Figure 2.1) (Mansfield and Briarty, 1991; Jürgens *et al.*, 1994). *Arabidopsis* embryogenesis is not especially typical of all higher dicotyledonous plants, but its genetic tractability has led to the unraveling of molecular mechanisms that control cell patterning and axis development, and many of these are likely to be conserved between plant species.

These molecular control mechanisms are the focus of this chapter. We consider what is known about the genes necessary for correct embryogenesis and the signaling systems that regulate the temporal and spatial expression of those genes. We do not consider the development of the endosperm, which, although it may influence embryogenesis, is not critical for it, at least at the earliest stages of development. The endosperm is considered in detail in Chapter 3. Also, we are unable to describe the functions of all genes known to be involved in the embryogenic process. Rather, we focus on hormone signaling systems (especially but not exclusively auxin signaling) and how these are used to activate genes in particular tissues as well as how they are themselves influenced by the functions of specific genes to create functional regulatory networks.

Cellular Events

Because mutational screens depend on the identification of abnormal phenotypes, it is appropriate to summarize briefly knowledge of the cellular events of *Arabidopsis* embryogenesis before discussing molecular events. Embryogenesis begins with the fertilization of the haploid egg cell with one

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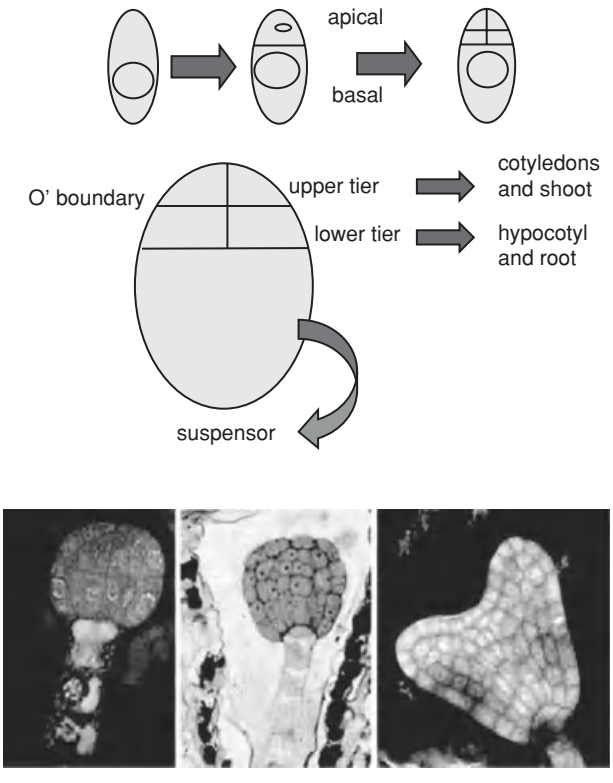

Figure 2.1 *Upper panel,* Diagrammatic representation of early events in *Arabidopsis* embryogenesis. The zygote undergoes an asymmetrical transverse division to form a small apical cell and a larger, more vacuolated basal cell. The basal cell divides to form the suspensor, while the apical cell divides transversely to form upper and lower tiers that develop into the cotyledons and shoot apical meristem and the hypocotyl and root. *Lower panel,* Developing globular *(left, center)* and heart *(right)* stage embryos of *Arabidopsis,* showing the upper cells of the suspensor. (For color detail, see color plate section.)

haploid sperm cell, to create the diploid zygote. As illustrated in Figure 2.1, the zygote divides asymmetrically to form the apical and basal daughter cells with different sizes and cytoplasmic densities (Mansfield and Briarty, 1991). This process defines establishment of the embryonic apical-basal axis, which represents the earliest patterning event in plant embryogenesis. However, it is evident that polarity already exists in the embryo sac, wherein the egg cell and synergids are located at the micropylar end, and the antipodal cells are found at the chalazal end. The egg cell itself also exhibits polarity before fertilization; it has a large vacuole at its micropylar end, whereas the chalazal end is relatively cytoplasmic (Schulz and Jensen, 1968; Mansfield and Briarty, 1991).

The first division of the fertilized egg cell reinforces this polarity. Two daughter cells, an apical cell and a basal cell, adopt different developmental fates in later embryogenesis. The apical cell of the embryo proper divides vigorously and forms most of the embryo. Through perpendicular shifts during three successive rounds of cell division, the apical daughter cell gives rise to an eight-cell embryo proper (defining the octant stage) comprising an apical and central embryo domain. The apical domain, composed of the four uppermost apical cells of the embryo proper, remains largely isodiametric before the initiation of the shoot meristem and most of the cotyledons. The central domain, consisting of the four lower cells of the embryo proper, undergoes divisions that are oriented

either parallel or perpendicular to the apical-basal axis, forms the hypocotyl and root, and contributes to part of the cotyledons and the root meristem. By contrast, the basal cell of the zygote divides only horizontally (perpendicular to the apical-basal axis) and to form a filamentous structure: the suspensor and its uppermost cell, the hypophysis. The hypophysis undergoes a sequence of reproducible divisions giving rise to part of the primary root meristem, comprising the quiescent center (QC) and the central (columella) root cap initials (Scheres *et al.*, 1994).The embryonic suspensor pushes the embryo into the lumen of the ovule and provides a connection to the mother tissue, which facilitates nutrition of the embryo proper.

The most basal suspensor cell enlarges dramatically and has abundant contact with surrounding maternal tissues, likely facilitating the supply of nutrients to the embryo. The suspensor appears to have many different functions: it physically projects the embryo into the endosperm and provides both a conduit and a source of hormones and nutrients for the developing embryo. Perhaps the clearest difference in fate between the embryo proper and suspensor is seen as the programmed cell death of the suspensor, which is correspondingly not present in mature seeds (Yeung and Meinke, 1993).

The main elements to be positioned along the apical-basal axis in the *Arabidopsis* embryo are the shoot apical meristem (SAM), cotyledons, hypocotyl, radicle, and root apical meristem. The establishment of the apical-basal axis, in the early stage of embryogenesis, and the radial axis comprising the concentric cell layers of the embryo is crucial for subsequent plant growth and development. The mechanisms regulating these processes are discussed in detail subsequently.

Genes and Signaling – the Global Picture

A range of approaches have been adopted over the past few decades to understand better the genetic basis of embryo development. Most functional analysis of genes has derived from mutational screens and subsequent gene analysis, including more specialized screens such as promoter trapping (Topping *et al.*, 1994). Mutation screens (typically using chemical mutagens such as ethyl methanesulfonate, which induce point mutations, or insertional mutagenesis via T-DNA or transposon insertion into the genome) have proved invaluable for dissecting gene function and form the mainstay of functional analysis. Promoter trapping can identify genes expressed during embryogenesis on the basis of the activation of a promoterless reporter gene in embryonic cell types and can generate mutations that inform gene function. More recently, transcription profiling techniques have been used to study embryo development, aided by the use of laser-capture microdissection (LCM) (Casson *et al.*, 2005; Spencer *et al.*, 2007). This latter technique allows the isolation of small cell clusters, even from specific cellular domains of globular stage embryos of *Arabidopsis*, for RNA extraction and amplification; this overcomes the technical problem faced for many years of not being able to collect sufficient RNA from the youngest embryos for cDNA library construction and analysis. In combination with microarray analysis or RNA sequencing, this method allows high-throughput and high-resolution transcriptional profiling of the developing embryo.

Despite the relative structural simplicity of the *Arabidopsis* embryo, evidence suggests that a large proportion of the genome is active during embryogenesis. The sequencing of the *Arabidopsis* genome has allowed the development of high-throughput gene expression platforms, such as DNA microarray chips, for the transcriptional profiling of the developing embryo. Microarray analysis of RNA samples from apical and basal tissue domains of globular-stage, heart-stage, and torpedo-stage embryos, following LCM (Figure 2.2), revealed that ~75% of the *Arabidopsis* genome on the ATH1 microarray chip is expressed at one or more tissues analyzed (i.e., up to ~17,000 genes) (Casson

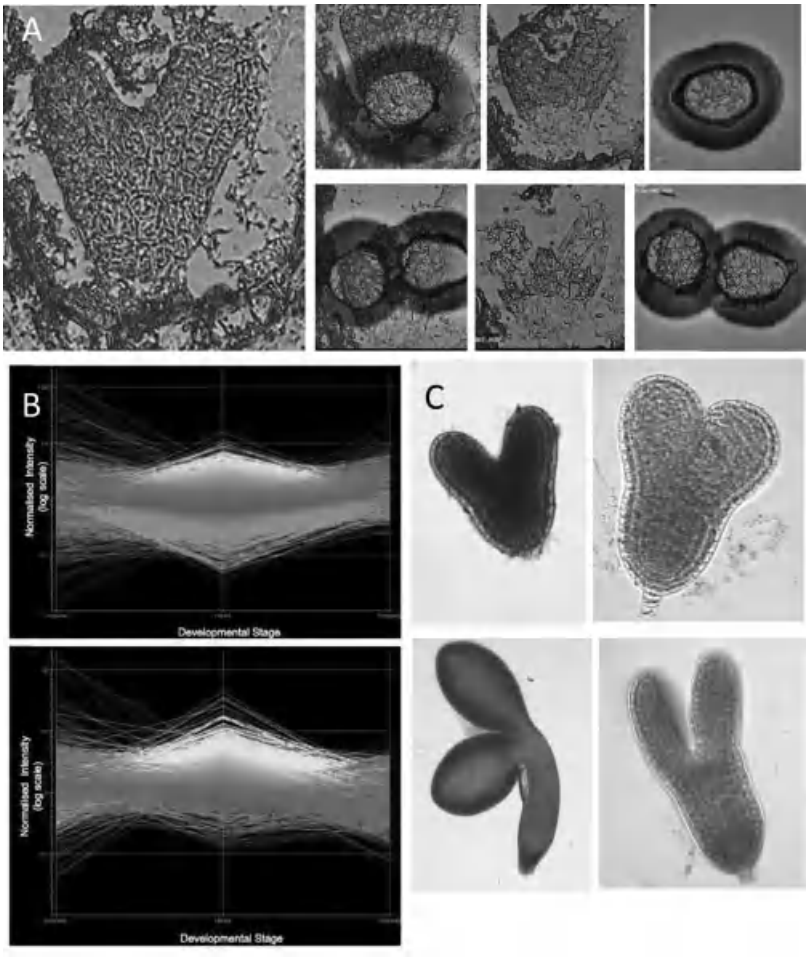

Figure 2.2 *A,* Laser-capture microdissection of cryosectioned heart stage embryo. *B,* Bioinformatics analysis of microarray data from genes expressed in apical *(upper panel)* and basal *(lower panel)* cells of developing embryos. *C,* Promoter-GUS fusion expression patterns of embryonically expressed genes identified by LCM. See Casson *et al.* (2005) and Spencer *et al.* (2007) for further detail. (For color detail, see color plate section.)

et al., 2005; Spencer *et al.*, 2007). This finding is broadly comparable with the proportion of genes found to be expressed in the primary root of *Arabidopsis* (Birnbaum *et al.*, 2003) and illustrates the high level of transcriptional activity during embryo development. The transition from the globular to the heart stage is associated in particular with an upregulation of genes involved in cell cycle control, transcriptional regulation, and energetics and metabolism. The transition from heart to torpedo stage is associated with a repression of cell cycle genes, as cell division slows in favor of cell enlargement, and genes encoding storage proteins are upregulated, together with pathways of cell growth, energy, and cell wall metabolism. The torpedo-stage embryo shows strong functional differentiation in the root and cotyledon, revealed by the distinct classes of genes expressed in these tissues. The transcriptional differences were greater between temporal stages of development than between different tissues of embryos at a given stage, suggesting rapid changes in expression

of many genes as development proceeds. Nevertheless, there are clear transcriptional differences between apical and basal regions of embryos at all stages analyzed (Spencer *et al.*, 2007). Le *et al.* (2010) also performed microarray analysis on developing *Arabidopsis* seeds, in part using LCM to isolate RNA from whole developing embryos and other tissues, and found similar numbers of genes expressed (16,000) during seed development; many are stage-specific, in agreement with the data of Casson *et al.* (2005) and Spencer *et al.* (2007).

These results are consistent with older work, based on RNA hybridization studies in tobacco (*Nicotiana tabacum*), which suggested that although populations of transcripts are organ-specific, 60%–77% of plant genes are expressed in heterologous organs (Goldberg, 1988). Goldberg *et al.* (1989) demonstrated that distinct mRNA sets are temporally regulated during embryogenesis, with expression restricted to specific developmental stages, and the LCM data support this global view of large-scale transcriptional changes during embryo development. More recent analysis has shown that, in contrast to animal embryogenesis, in which there is strong maternal control over embryo transcript abundance and development, in *Arabidopsis* the zygotic genome is the principal regulator of early embryogenesis (Nodine and Bartel, 2012). microRNAs (miRNAs) are required to prevent premature expression of transcription factors that, if derepressed, lead to embryo lethality (Nodine and Bartel, 2010).

Coordination of Genes and Cellular Processes: a Role for Hormones

The patterning of cellular organization in the three-dimensional structure of the developing embryo requires the spatial and temporal expression of genes that are revealed by the above-described transcriptomic studies. This control of gene expression is itself presumed to be patterned by signaling systems, in particular (but not necessarily only by), mobile plant hormones, which provide a chemical regulatory framework. The best-characterized hormone system that has been shown to influence embryogenesis, not only in *Arabidopsis* but also in other species, is auxin. The recognition of the importance of auxin in providing positional information in the developing embryo has been instrumental in allowing us to understand better the molecular mechanisms through which specific genes act.

Auxin exists in several forms within the cell, and although its biosynthetic pathway is still incompletely understood, the most abundant biologically active form is free (nonconjugated) indole-3-acetic acid (IAA). There is a tight control over auxin homeostasis to regulate cellular concentration (Woodward and Bartel, 2005), although relatively little is known about specific mechanisms during embryogenesis. It is known, however, that the *YUCCA* gene, which encodes a flavin monooxygenase, is necessary both for auxin biosynthesis and for embryo development (Cheng *et al.*, 2007). It has been found more recently that induction of the SHORT-INTERNODES/STYLISH (SHI/STY) family member *STY1* results in increased transcript levels of the *YUCCA4* gene and higher auxin levels and auxin biosynthesis rates in *Arabidopsis* seedlings (Eklund *et al.*, 2010). Evidence indicates that *STY1*, a likely transcription factor, activates genes involved in auxin biosynthesis through promoter binding, with a role in the formation or maintenance, or both, of the shoot apical meristem, possibly by regulating auxin levels in the embryo.

Synthetic analogues, such as naphthalene-1-acetic acid (NAA) and 2,4-dichorophenoxyacetic acid (2,4-D), have been used experimentally to investigate auxin-mediated effects on development, owing to their different properties; for example, in contrast to IAA or 2,4-D, NAA can readily diffuse into cells without the need for uptake transporters. IAA is a weak acid, and because the extracellular pH is lower than the cytoplasmic pH, it is partially protonated in the extracellular

matrix, which increases its membrane permeability, and it diffuses into cells slowly (Blakeslee *et al.*, 2005). However, the AUX1/LAX family of influx carriers promotes rapid auxin uptake into cells (Bennett *et al.*, 1996; Swarup *et al.*, 2008), whereas the PIN-FORMED (PIN) auxin transporters, in association with ATP-binding cassette-like transporters such as AVP1 and PGPs (Li *et al.*, 2005; Mravec *et al.*, 2008), facilitate the efflux of negatively ionized auxin.

Inside the cell, auxin mediates its effects by inducing the derepression of auxin-responsive genes. Such genes contain auxin-responsive *cis*-acting elements in their promoter regions, which bind transcription factors termed auxin response factors (ARFs). *Arabidopsis* contains 23 such proteins, and these typically dimerize and may act as positive or negative regulators of transcription (Reed, 2001). ARF function is repressed by interacting Aux/IAA genes, which, in the absence of auxin, bind them but do not bind DNA. In *Arabidopsis*, there are 29 ARF family members (Reed, 2001; Li *et al.*, 2006). In the presence of auxin, the Aux/IAA proteins are degraded by the proteosome via a mechanism involving the SCF ubiquitin ligase complex, whereby auxin acts as a "molecular glue" to bring together the TIR1 F-box protein of SCF and the target Aux/IAA protein to promote its ubiquitination and subsequent degradation. This mechanism releases the ARF complex to activate (or in some cases block) gene transcription.

The role of auxin as a mediator of cellular patterning in embryogenesis has been suspected for many years now. Liu *et al.* (1993) first demonstrated that auxin transport inhibitors induced developmental defects in embryogenesis, specifically in cultured zygotic embryos of the *Arabidopsis* relative, *Brassica juncea*. Inhibition of polar auxin transport at the globular stage led to the formation of embryos lacking bilateral symmetry at the heart stage. Embryos developed with fused and collar-like cotyledons instead of two distinct lobes, which phenocopied known auxin transport-defective mutants *pin1* (Okada *et al.*, 1991) and *gnom* (Steinmann *et al.*, 1999).

Similarly, Hadfi *et al.* (1998) found that when auxin was supplied to the same *B. juncea* embryos, ball-shaped or cucumber-shaped embryos resulted, possibly because embryos flooded with exogenous auxin are unable to establish the auxin gradients that are essential for normal morphogenesis. Treatment with the antiauxin PCIB inhibited cotyledon growth so that either only one or no cotyledons developed. Correct hypocotyl and radicle growth was also found to require auxin action and movement. When globular-stage embryos were treated with exogenous NPA, a polar auxin transport inhibitor, axis duplication resulted, whereas a later application produced split-collar or collar-like cotyledons. Similar results were found by Fischer *et al.* (1997) for morphogenesis of the embryo of the monocot wheat and for somatic embryos of Norway Spruce (*Picea abies*) (Hakman *et al.*, 2009). Mutations in the *AMINOPEPTIDASE M1* (*APM1*) gene of *Arabidopsis*, encoding a metalloprotease with affinity for and ability to hydrolyze NPA, lead to defects in embryonic and seedling patterning, PIN localization, and auxin transport (Peer *et al.*, 2009).

These results formed the basis for a model in which continuous auxin transport removes auxin from the area between the two emerging cotyledons and supplies the auxin back to the cotyledonary primordia. Auxin removal starts in the central apical region of the globular or early transition embryo, and continues asymmetrically across the apex of the embryo. Inhibition of auxin transport blurs the positional information that is created by its normally precise redistribution, resulting in increased cell division throughout the shoot apex. These findings indicate that auxin translocation is a prerequisite for the radial globular embryo to progress to the bilaterally symmetrical heart stage embryo.

Insight into the molecular mechanisms underpinning these observations came from genetic studies. A key feature of the way by which auxin orchestrates cellular patterning is its directional movement, which leads to the formation of gradients in its concentration. The PIN proteins are encoded by a multigene family, with eight members. Of the eight, PIN1, PIN2, PIN3, PIN4, and

PIN7 are localized to specific faces of the plasma membrane, which provides a mechanism for transport auxin in particular directions. PIN5, PIN6, and PIN8 are located in the endoplasmic reticulum (Mravec *et al.*, 2009), and endoplasmic reticulum localization is likely related to a role for these particular PINs in auxin homeostasis rather than intercellular transport.

The functional significance of PIN proteins in embryogenesis is further revealed by mutational studies. The *pin1* mutant exhibits embryonic defects similar to embryos treated with auxin transport inhibitors (Okada *et al.*, 1991; Liu *et al.*, 1993), and multiple mutants between *pin1*, *pin3*, *pin4*, and *pin7* exhibit a range of embryonic defects (Friml *et al.*, 2003). Immunolocalization of the PIN proteins shows that they have precise patterns in the developing embryo, and their correct function is necessary for correct auxin distribution, as revealed by the pattern of expression of the auxin reporter gene *DR5::GUS* or *DR5::GFP*, and correct development.

The results of this work can be summarized as follows: Once the egg cell has been fertilized and the zygote undergoes a first asymmetrical division, auxin accumulates in the apical cell, leading to the specification of this cell as the founder of the proembryo. This cell acts as an auxin sink, whereby auxin is transported from the adjacent basal cell by PIN7-dependent transport (PIN7 is polarly localized to the apical plasma membrane of the basal cell). This specification is disrupted in *pin7* and multiple *pin* mutants. During the globular stage of development, auxin production may occur in the apical domain of the embryo region. PIN1 basal localization is also established in the provascular cells, which may promote auxin transport basally, toward the developing root promeristem. At this time, the asymmetrical localization of PIN7 is reversed in the basal cells, promoting auxin transport out of the embryo and into the suspensor. PIN4 expression is induced in the basal region of the embryo, moving auxin in association with both PIN1 and PIN7. As a consequence, a new auxin concentration maximum is established in the hypophysis, specifying the founding cells of the root meristem (Figure 2.3).

A different experimental approach to understanding the role of auxin in embryogenesis has been described by Weijers *et al.* (2005). These authors manipulated auxin homeostasis in transgenic *Arabidopsis*, by tissue-specific expression of bacterial genes encoding the indoleacetic acid–tryptophan monooxygenase (*iaaM*) or indoleacetic acid–lysine synthetase (*iaaL*) involved in auxin biosynthesis

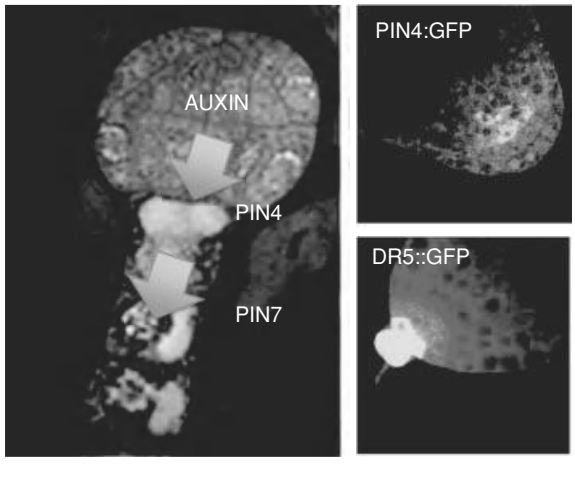

Figure 2.3 Auxin is translocated in the developing globular embryo from the apical region to the hypophysis via PIN4 and then from the hypophysis to the suspensor via PIN4 and PIN7. There is an auxin maximum (seen as DR5::GFP expression) at the basal region of the embryo. (For color detail, see color plate section.)

and conjugation. Despite the demonstrable experimental increases in auxin synthesis and conjugation, there was no discernible effect on either auxin gradients or morphological development of the embryos. This work suggests a strong buffering capacity of the auxin transport system in the developing embryo, and this was shown to be regulated principally by PIN1 and PIN4. Such a robust mechanism probably accounts in part at least for the strongly stereotyped and predictable cellular patterning seen in *Arabidopsis* embryogenesis.

Less information is available for roles for other hormones in the regulation of *Arabidopsis* embryogenesis or at least the early patterning mechanisms. Gibberellins (GA) and abscisic acid (ABA) have been known for many years to regulate later stages of seed development. During the later stages of embryogenesis, from heart stage onward, a biochemical differentiation of the embryo takes place, during which it accumulates chlorophyll (a marker for plastid maturation) and begins synthesis of storage lipids (triacylglycerols) in the plastid and seed storage proteins. In Brassicaceae such as *Arabidopsis*, the embryo is the main storage compartment for these metabolites, and the mature embryo is packed full of oil and protein bodies. GA and ABA play important roles in regulating seed maturation, in concert with sugar metabolism, and links with specific genes are becoming clear, mainly through genetic studies.

Details of seed maturation mechanisms are discussed elsewhere in this book (see Chapter 6), but some key points pertinent to embryogenesis are summarized here. Genes in *Arabidopsis* such as *LEC1*, *LEC2*, *FUS3*, and *ABI3* (part of the so-called LEC1/B3 transcription factor network) are key regulators of aspects of embryo development that include storage product synthesis and seed maturation. LEC1 (LEAFY COTYLEDON 1) was originally identified as a mutant defective in cotyledon identity, showing features of leaflike quality such as trichomes, but also exhibiting morphological defects earlier in embryogenesis, such as abnormal hypocotyl elongation, root pole shape, and shoot meristem organization (Meinke *et al.*, 1994; West *et al.*, 1994). The gene was cloned and found to encode a transcription factor subunit related to the HAP3 subunit of the CCAAT binding factor family (Lotan *et al*., 1998). *FUS3* and *LEC2* encode B3 domain transcription factors (Luerssen *et al*., 1998; Stone *et al.*, 2001). *fus3* mutants, similar to *lec1*, show both early and late embryonic phenotypes (Baumlein *et al.*, 1994; Keith *et al.*, 1994). *fus3* (named from the Latin for "purple," reflecting the ectopic anthocyanin accumulation in *fusca* seedlings) forms trichomes on cotyledons, is desiccation-intolerant, and is defective in storage product accumulation. The *FUS3* transcript, similar to *LEC1*, is preferentially localized to the protoderm (the embryonic epidermis) in early embryogenesis, consistent with the ectopic trichome and anthocyanin phenotypes (Tsuchiya *et al.*, 2004). It acts as a negative regulator of the anthocyanin-activating and trichome-activating *TRANSPARENT TESTA 1* (*TTG1*) gene during embryo development. The LEC2 protein is closely related to FUS3 and, similar to FUS3 and LEC1, plays a role in controlling cotyledon identity and storage product accumulation.

Transgenic expression of *LEC1*, *LEC2*, or *FUS3* leads to the activation of embryonic pathways in vegetative tissues, seen as the accumulation of triacylglycerols and seed storage proteins, and even somatic embryogenesis in vegetative tissues (Lotan *et al*., 1998; Santos Mendoza *et al.*, 2005; Casson and Lindsey, 2006; Stone *et al.*, 2008). LEC1 regulates *LEC2*, *FUS3*, and *ABI3* expression, although LEC2 can also activate *LEC1* and *FUS3*, and *lec2* mutants show reduced *ABI3* expression (To *et al.*, 2006; Baybrook and Harada, 2008). ABI3 is a transcription factor, representing the *Arabidopsis* orthologue of maize VP1, regulating seed maturation and preventing premature germination (vivipary) (Suzuki and McCarty, 2008). The embryo also acquires desiccation tolerance, in part at least through ABA signaling and the accumulation of the protective LATE-EMBRYOGENESIS ABUNDANCE (LEA) proteins (Hundertmark and Hincha, 2008). ABA signaling is now well known as a regulator of embryo maturation and acts via ABI3 (the *abi3* mutant is ABA-insensitive)

(Finkelstein *et al.*, 2002). The link between auxin and storage product accumulation is less clear, but we have shown that LEC1 effects are dependent on auxin and sucrose in *Arabidopsis* (Casson and Lindsey, 2006). Stone *et al.* (2008) also showed that transgenic overexpression of *LEC2* leads to an upregulation of auxin biosynthesis via an induction of *YUCCA* gene expression (*YUC2* and *YUC4*). Auxin upregulates *FUS3* expression, and FUS3 is a positive regulator of ABA levels (Gazzarini *et al.*, 2004). Auxin also represses *LEC2* expression (Casson and Lindsey, 2006), providing further evidence of a link between these transcription factors, auxin, and embryonic identity and morphogenesis.

Gibberellins also play a role in controlling embryogenesis. FUS3 and LEC2 negatively regulate GA synthesis, by repressing the GA synthesis gene *GA3ox2* (Curaba *et al.*, 2004; Gazzarini *et al.*, 2004). LEC2 directly activates *AGAMOUS-LIKE 15* (*AGL15*), which is itself an activator of the catabolic gene *GA2ox6*, leading to a reduction in GA levels (Wang *et al.*, 2004). Overexpression of *AGL15* leads to enhanced somatic embryogenesis in *Arabidopsis* (Harding *et al.*, 2003). Reduced GA levels generally appear to be required for the maintenance of the embryonic development program, whereas increased GA levels (and decreased ABA) promote germination.

Repression of the LEC1/B3 transcription factors in postembryonic (vegetative) tissues is a normal feature of plant development (Figure 2.4). This repression is in part regulated by chromatin remodeling, mediated by the PICKLE (PKL) protein. *pkl* mutants show ectopic embryogenesis and storage oil accumulation, through derepression of *LEC* genes (Ogas *et al.*, 1997, 1999; Dean Rider *et al.*, 2003). The *pkl* phenotype is repressed by exogenous GA and promoted by treatment with GA inhibitors, illustrating the link between *LEC* gene function, GA, and embryogenesis. PKL is also required for the repression of the auxin response factors ARF7 and ARF19 (Fukaki *et al.*, 2006). Three *VP1/ABI3-LIKE* (*VAL*) genes, encoding B3-related transcription factors associated with chromatin factors, also repress *LEC1*, *ABI3*, and *FUS3* expression, as part of a likely chromatin-mediated repression system in vegetative tissues (Suzuki *et al.*, 2007). Similarly, the ectopic activation of embryonic genes by a gain-of-function mutant of *LEC1*, designated the *turnip* mutant, showed an enhanced penetrance of the phenotype in the presence of GA inhibitors (Casson and Lindsey, 2006). A link was also found with auxin – the *tnp* phenotype was enhanced in the

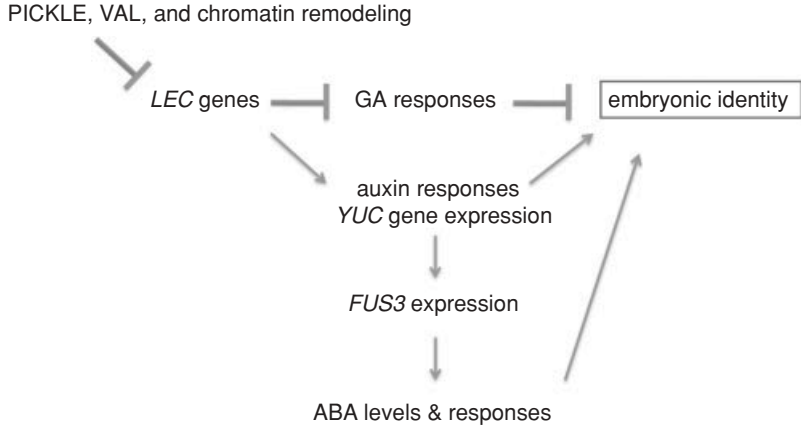

Figure 2.4 *LEC1/B3* genes are repressed in nonembryonic tissues by chromatin-associated factors, including PICKLE and VAL proteins. *LEC* genes appear to promote embryonic identity by repressing GA responses, promoting ABA responses, and may work via auxin.

presence of exogenous auxin, and the *tmp* mutant showed enhanced auxin responses, revealed as an increased level of expression of the auxin-regulated *POLARIS* gene and the *IAA2::GUS* reporter gene. Because *LEC2* expression was not upregulated in *tmp*, the high auxin phenotype was probably not mediated by *LEC2*. However, *LEC1* expression appears not to be regulated by auxin, GA, or cytokinin (Casson and Lindsey, 2006).

A further link between auxin and GAs has been found in relation to embryo development. Willige *et al.* (2010) showed that auxin transport is reduced in GA-deficient mutants, and this is due to reduced PIN protein levels. For PIN2, at least, this reduction is due to vacuolar degradation. Morphologically, this is associated with defective cotyledon development. GA has been shown to influence cotyledon development previously, with the GA signaling-deficient *spindly* mutants having only one cotyledon or fused cotyledons (Hartweck *et al.*, 2002; Silverstone *et al.*, 2007), which is also seen in auxin transport mutants, as described previously.

Relatively little known is about the role of cytokinins during embryogenesis. Cytokinin represses both *LEC2* and *FUS3* transcription (Casson and Lindsey, 2006), and this may be partly due to the negative regulation of auxin transport and responses by cytokinin (Dello Ioio *et al.*, 2008; Ruzicka *et al.*, 2009). Cytokinins are known to regulate cell division, through CYCLIN D3 activation (Riou-Khamlichi *et al.*, 1999). The *ALTERED MERISTEM PROGRAM 1* (*amp1*) mutant, defective in a carboxypeptidase, has enhanced cytokinin levels and associated defects in cotyledon development (Chaudhury *et al.*, 1993). Some alleles also show cell division abnormalities in the basal region of the embryo, and AMP1 plays a role in antagonizing auxin responses necessary for correct embryo and root development, mediated by the ARF5 MONOPTEROS (MP). *amp1* can suppress the *mp* mutant phenotype, by restricting cell number in the embryo (Vidaurre *et al.*, 2007). Repression of cytokinin synthesis by overexpressing genes encoding cytokinin oxidase/dehydrogenease (CKX) enzymes leads to defective embryo size but, surprisingly, larger rather than smaller embryos (Werner *et al.*, 2003). Paradoxically, expression of a cytokinin reporter gene shows activity in the hypophysis, suspensor, and root pole of developing *Arabidopsis* embryos (Müller and Sheen, 2008). This is the area of auxin accumulation in the embryo, as discussed previously, and the data suggest that auxin antagonizes cytokinin responses in this part of the embryo by transcriptional activation of the cytokinin-repressing *ARABIDOPSIS RESPONSE REGULATOR* genes *ARR7* and *ARR15*. Mutation of these genes or ectopic cytokinin signaling in the basal region of the embryo results in a defective root stem-cell system.

Genes and Pattern

So far we have focused the discussion on signaling systems that affect embryogenesis, and an important question relates to the genes that are regulated by these systems and how they exert their cellular effects. Genes essential for correct embryogenesis have been identified predominantly through mutational screens, leading to a classification of mutants as either embryo-lethal or seedling-lethal. The distinction is based on the definition that embryo-lethals arrest at some point during embryogenesis, but before germination, whereas seedling-lethal mutants germinate but arrest at the seedling stage, failing to go on and flower. Meinke's laboratory has been instrumental in characterizing and establishing a database of embryo-lethal mutants, from early work dating back to the 1970s (Meinke and Sussex, 1979), as discussed in Chapter 1. The framework for thinking about the genetic mechanisms regulating pattern formation during *Arabidopsis* embryogenesis gained momentum through screens for and analysis of seedling-lethal mutants and has to a large extent been driven by the laboratory of Jürgens.

A mechanistic understanding of genes required for embryonic and seedling patterning has been made easier by the rapid improvement of knowledge of signaling pathways in plants because many key genes, considered as patterning genes, have an impact on these pathways by acting through them as well as being activated in response to them. Embryonic development, similar to other aspects of development, involves complex gene and signaling networks that are characterized by often counterintuitive feedback loops and interactions, in which each component likely has an effect on all other components, at some level. A "systems biology" approach to understanding embryonic gene and signaling networks is still in its infancy and is expected to be increasingly explored as computational modeling approaches develop further. However, to date, most of our knowledge comes from the more reductionist genetic approach, with all its attendant strengths and weaknesses.

In 1991, Mayer *et al.* published an important article that described an approach to identify patterning genes in *Arabidopsis*, through the screening for seedling-lethals that "lack" specific pattern elements (essentially the shoot and root meristems and hypocotyl that define the apical-basal axis and concentric tissue layers defining the radial axis). Although one possible outcome of such an approach would be to identify sets of transcription factors (e.g., that specify the identity of such pattern elements), genes encoding a wide range of protein functions, from transcription factors to vesicle transport proteins to enzymes, have been elucidated from ensuing studies of such mutants. Perhaps the main value of the approach has been to encourage a consideration of the relevance of the nature of "pattern elements" in plants, given that plants are organisms that exhibit enormous plasticity of development as a consequence of their intercellular communication systems, totipotency, and lack of cell migration.

One gene identified in both embryo-lethal and seedling-lethal mutants screens is *EMB30* (Meinke, 1985), also known as *GNOM* (Mayer *et al.*, 1991, 1993). The mutant embryo can germinate to form an abnormal seedling that may lack apical meristems and in extreme cases develop as a ball of cells lacking polarity and misexpressing genes that are normally expressed in a polar fashion, such as *POLARIS* (Topping and Lindsey, 1997). Sibling seedlings of the same genotype typically show a range of phenotypes, from "golf tee"–like seedlings with fused cotyledons to apolar balls. The embryos show defects from the first division of the zygote, which often lacks the asymmetry typical of wild-type, with subsequent defects in cell division and expansion (Mayer *et al.*, 1993) and in cell wall organization (Shevell *et al.*, 2000).

Cloning of the gene revealed that GNOM is a member of the ARF-GEF family of proteins (ADP-ribosylation factor guanine nucleotide exchange factors), similar to yeast SEC7 (Shevell *et al.*, 1994; Busch *et al.*, 1996). These proteins are reversibly recruited to membranes required, as dimers, for membrane tethering and vesicle fusion (Anders *et al.*, 2008). The fungal toxin Brefeldin A (BFA) is a specific inhibitor of both ARF GEFs and polar auxin transport, and Steinmann *et al.* (1999) showed that BFA-sensitive GNOM is required for the correct polar localization of the PIN1 auxin efflux carrier during embryogenesis. BFA treatment leads to the cytosolic accumulation of PIN1, and this observation has formed the basis of the model for the regulation of PIN localization and dynamics by endocytic recycling, in a pathway dependent on GNOM activity (Geldner *et al.*, 2001, 2003). Auxin transport inhibitors such a triiodobenzoic acid also inhibit PIN localization by disrupting endocytic recycling. There appear also to be distinct ARF-GEF activities translocating vesicle cargoes to the apical versus the basal sides of the cell, and evidence suggests that GNOM has a specific role in vesicle transport to the basal membrane (Kleine-Vehn *et al.*, 2008).

Mutation of the *PINOID* (*PID*) gene, encoding a kinase, also leads to defective PIN localization, and defects in embryogenesis are seen in transgenic plants overexpressing *PID*. In contrast to *gnom* mutants, which show complete disorganization of PIN localization, *PID* overexpressors exhibit a shift from basal to apical PIN1 localization, leading to altered auxin gradients and mis-specification

of the hypophysis (Friml *et al.*, 2004). These results suggest that PIN protein phosphorylation is important in determining the fate of the cell to which it is translocated. Quadruple mutants for *PID* and its three most related kinase gene family members (*PID2*, *WAG1*, *WAG2*) exhibit a loss of cotyledon development (Christensen *et al.*, 2000; Friml *et al.*, 2004; Cheng *et al.*, 2008). The PP2A phosphatase anatagonizes *PID* function (Michniewicz *et al.*, 2007).

The asymmetrical division of the embryo defines a critical step in *Arabidopsis* embryogenesis, by creating apical and basal domains with distinct fates. Although suspensor development requires expression of the YODA (YDA) MAP kinase kinase kinase (Lukowitz *et al.*, 2004; Bayer *et al.*, 2009), an early feature is the differential expression between apical and basal regions of the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) transcription factor genes (Haecker *et al.*, 2004). Both *WOX2* and *WOX8* genes are expressed in the zygote, and subsequently *WOX2* expression is found in the apical cell, whereas *WOX8* is expressed in the basal cell. *WOX9* appears to be expressed after the zygotic division, possibly in both apical and basal cells, and *WOX5*, a marker of the QC, is expressed early in the hypophysis, showing its early specification. *WOX8* expression is necessary for *WOX2* expression in the proembryo, and *WOX* genes are also required for correct proembryo development through correct *PIN1* expression and embryonic auxin distribution (Breuninger *et al.*, 2008).

Hypophysis specification also requires auxin signaling and is defective in embryos carrying mutations in *MP*, *PIN* genes (Friml *et al.*, 2003), *AXR6* (Hellman *et al.*, 2003), or genes encoding enzymes in auxin biosynthesis such as *YUC* genes (Cheng *et al.*, 2007) or tryptophan aminotransferases (Stepanova *et al.*, 2008). POLTERGEIST (POL) and the related POLTERGEIST-LIKE1 (PLL1) are phosphatases also necessary for correct hypophysis division and, similar to MP, for vascular development (Song *et al.*, 2008), perhaps suggesting an inductive effect from cells within the embryo.

How is the boundary between apical and basal domains established in the proembryo? One gene with a role in this process is *HANABA TARANU* (*HAN*) (Nawy *et al.*, 2010). *HAN* encodes a GATA factor, in which loss of function causes a shift in gene expression from the basal to the apical region of the proembryo, including *WOX5*; *SUCROSE TRANSPORTER3* (normally expressed in the suspensor); reduced *SHORTROOT* (normally in or adjacent to the hypophysis); and *PIN1* and *PIN7*. These changes in gene expression lead to the ectopic formation of a root meristem in the central region of the globular-shaped *han* embryo. *HAN* activity appears to be necessary for the establishment of correct basal gene expression and auxin distribution required for apical-basal pattern formation.

A second gene to come out of the screen by Mayer *et al.* (1991) and required for the correct development of the *Arabidopsis* apical-basal axis is *MONOPTEROS*. Mutation of *MP* leads to the development of seedlings with no, or a very short, hypocotyl and no primary root, with only a "basal peg" in the position below the cotyledons (Berleth and Jürgens, 1993). Seedlings also typically have abnormal vasculature in the cotyledons, with some strong alleles lacking veins almost completely. These defects can be traced back to early embryogenesis, during which the octant stage has four rather than two tiers of cells, defective cell patterning (especially in the central cells of the vascular primordium) is seen at the heart stage, and asymmetrical cotyledon development occurs. As previously mentioned, *MP* encodes ARF5 required for the activation of auxin response genes, further linking embryo patterning to auxin signaling (Hardtke and Berleth, 1998). MP/ARF5 interacts with the inhibitory Aux/IAA12 protein BODENLOS (BDL) (Hamann *et al.*, 2002), and similar to the loss-of-function *mp* mutant, the gain-of-function *bdl* mutant also exhibits defective apical-basal patterning and root meristem formation in the embryo (Hamann *et al.*, 1999). NONPHOTOTROPIC HYPOCOTYL4 (NPH4) is also an ARF (ARF7), closely related to

MP. NPH4 dimerizes with MP and, similar to MP, homodimerizes and is required for embryonic axis formation (Hardtke *et al.*, 2004). The dimerization of ARFs and interactions with various Aux/IAA proteins likely account for the diversity of auxin responses in different cell types, each of which might be expected to express different combinations of these regulators.

MP and BDL induce auxin transport from a subgroup of embryonic cells in the basal domain into the hypophysis (Weijers *et al.*, 2006). Downstream targets of these transcriptional regulators are beginning to be identified through approaches such as microarray experiments on mutants or overexpressors. For example, following dexamethasone-induction of *MP* expression, Schlereth *et al.* (2010) identified numerous known auxin signaling genes, including basic helix-loop-helix transcription factors *TMO5* and *TMO7* that are expressed in embryonic cells at the root pole. These are required for *MP* function in embryonic root development, and TMO7 is a mobile protein that moves from its site of synthesis in the embryo to its site of action in the hypophysis.

Another target of MP is the AP2 transcription factor DORNRÖSCHEN (DRN) (Cole *et al.*, 2009). Chromatin immunoprecipitation shows direct binding of MP to the *DRN* promoter, controlling its expression in the apical region and cotyledons of the developing embryo. Because DRN and the related DRN-LIKE also interact with the brassinosteroid signaling basic helix-loop-helix protein BIM1, these proteins provide evidence of molecular crosstalk between auxin and brassinosteroids to control embryonic patterning (Chandler *et al.*, 2009).

Further information on the auxin-transcription factor network is revealed by the *topless* (*tpl*) mutant. Severe mutant alleles of *TPL* exhibit a disruption of apical development of the *Arabidopsis* embryo, with homeotic transformation of the shoot into a root (Long *et al.*, 2002, 2006). TPL is a WD-40 repeat-containing protein that interacts with BDL and probably forms part of a protein complex, as suggested by biomolecular fluorescence complementation (BiFC) assays (Szemenyei *et al.*, 2008). *bdl* mutants cannot fully repress auxin responses in a *tpl* background, demonstrating the functional significance of this interaction. In addition, TPL has been found to regulate the *PLT* genes, and ectopic expression of the PLT transcription factors leads to the conversion of shoot to root (Smith and Long, 2010). It was also shown that the class III homeodomain-leucine zipper (HD-ZIP III) transcription factors control embryonic apical fate and are themselves sufficient to drive the conversion of the embryonic root pole into a second shoot pole. There is evidence of an antagonistic relationship between the PLT and HD-ZIP III genes in specifying the root and shoot poles.

The *POPCORN* (*PCN*) gene has been identified more recently that is also required for correct integration of the auxin signaling pathway with embryogenesis and the correct development of shoot and root apical meristems (Xiang *et al.*, 2011). *PCN*, similar to *TPL*, encodes a WD-40 protein, and the *pcn* mutant embryos exhibit abnormal cellular patterning after the two-cell stage of development, with defective bilateral symmetry (cotyledon morphogenesis), hypophysis, and columella development. The mutant also has numerous postembryonic defects suggestive of abnormal meristem function. Consistent with this, the mutant exhibits defective expression of meristem genes, whereby *WUSCHEL* (*WUS*), *CLAVATA3* (*CLV3*), and *SHOOTMERISTEMLESS* (*STM*) are upregulated in the SAM. Auxin responses (as determined by patterns of *DR5::GFP* expression) are altered in the developing embryo and associated with altered localization of PIN1 and expression of *PIN7*, *MP*, *PLT1*, *PLT*2, *PLT3*, *BDL*, and *WOX5*. It is suggested that PCN, similar to TPL, acts as a repressor of auxin responses, although there is no indication of a direct physical interaction with BDL, MP, or TPL. Cotyledon development also requires PIN1 and PID function (Furutani *et al.*, 2004) and *ENHANCER OF PINOID* (Treml *et al.*, 2005).

As discussed earlier, laser-capture microdissection in combination with microarray analysis has led to the identification of thousands of genes that are differentially expressed in apical and basal

domains from globular stage onward (Casson *et al.*, 2005; Spencer *et al.*, 2007). One gene that emerged from this screen and has been characterized in some detail is *MERISTEM-DEFECTIVE* (*MDF*) (Casson *et al.*, 2009). Identified on the basis of its expression in the basal domain of the heart-stage embryo, it is required for correct divisions of the basal domain of the globular embryo and subsequent root meristem development and function. The encoded protein is an RS domain protein, with a likely role in regulation of transcription or RNA processing. It is required for correct auxin distribution and *PIN2* expression in the embryo and for *PLT*, *BABY BOOM* (*BBM*), *SCARECROW* (*SCR*), and *SHORTROOT* (*SHR*) expression. It is also required for correct expression of the apical genes *WUS* and *CLV3* and subsequent shoot development. Transgenic overexpression of *MDF* leads to ectopic meristem activity and accumulation of embryonic triacylglycerols, suggesting it acts upstream of multiple embryonic pathways, although it is not itself regulated by auxin. BBM is a member of the PLT protein family, and its overexpression leads also to activation of embryonic pathways in vegetative cells (Boutilier *et al.*, 2002); this may be at least part of the mechanism by which MDF acts.

Once the apical domain of the embryo has been initiated, early activation of the expression of transcription factors leads to the specification of the future SAM. Essential in the process are two types of homeodomain proteins: KNOTTED-related STM (Barton and Poethig, 1993; Long *et al.*, 1996) and WUS (Laux *et al.*, 1996; Mayer *et al.*, 1998). Loss-of-function mutation of either gene leads to a failure of SAM formation; therefore, both genes function as positive regulators of the stem cell population in the developing embryo. In later stages of plant development, the *CLAVATA* genes work in antagonism to WUS, as negative regulators of cell production in the SAM-*clv* mutants are characterized by an excessive number of cells in the SAM (Clark *et al.*, 1993, 1995; Lenhard *et al.*, 2002). CLV1 is a predicted receptor kinase that in association with CLV2 interacts with the CLV3 peptide (Ogawa *et al.*, 2008), and a regulatory loop is established between WUS and the CLV complex to regulate the size of the SAM. It is also possible that WUS has an as yet unclear role in embryogenesis because its overexpression can induce somatic embryo formation on vegetative structures (Zuo *et al.*, 2002). ZWILLE (ZLL) is required to maintain meristem cell identity in the SAM and interacts with STM (Moussian *et al.*, 1998), as does CUP-SHAPED COTYLEDON (CUC) (Aida *et al.*, 1997, 1999), which is also linked to auxin signaling (Aida *et al.*, 2002; Furutani *et al.*, 2004). ZLL is a member of the ARGONAUTE protein family and is required to maintain HD-ZIP III expression levels in the SAM (Liu *et al.*, 2009). Ectopic expression of the HD-ZIP III genes *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) in the basal domain of the heart-stage embryo leads to defects in root development, and this is regulated by a SERRATE-miRNA165/166–dependent pathway (Grigg *et al.*, 2009). *HD-ZIP III* genes also interact with the *KANADI* genes (encoding GARP family transcription factors) to regulate *PIN1* expression and embryonic pattern (Izhaki and Bowman, 2007).

These genes mediate their effects through control of hormone responses. STM and the related KNOX family transcription factors repress GA signaling in the SAM. *NTH15* of tobacco encodes a *KNOX* protein, which downregulates the expression of the GA biosynthetic gene *GA20-OXIDASE* in tobacco meristems. It binds directly to the *GA20-OXIDASE* gene promoter, reducing the levels of active GA (Sakamoto *et al.*, 1999). Similarly, KN1 of maize directly binds and activates the promoter of the GA catabolic gene, *GA2OX* (Bolduc and Hake, 2009). Cytokinin has been known for many years to promote shoot meristem formation in plant tissue cultures, and both *STM* and *KNOX* genes are upregulated in cytokinin-overexpressing transgenics (Rupp *et al.*, 1999), as is *WUS* (Bäurle and Laux, 2005; Gordon *et al.*, 2009). *WUS* expression is also induced early in *Arabidopsis* somatic embryogenesis in response to auxin and is necessary for this developmental process (Su *et al.*, 2009). Overexpression of *WUS* can lead to ectopic somatic embryogenesis in vegetative tissues (Zuo *et al.*,

2002). WUS itself has been shown to regulate cytokinin response directly by repressing the *ARR* genes, which themselves negatively regulate cytokinin responses (Leibfried *et al.*, 2005), revealing a cytokinin feedback loop to control stem cell numbers in the SAM. For a more detailed discussion of the molecular development of the SAM, see Simon (2004).

Superimposed on the apical-basal axis during embryogenesis is patterning of the radial axis. In plants, this patterning is seen as concentric rings of tissue, comprising the dermal, ground, and vascular tissues. Radial pattern is established very early in *Arabidopsis* embryogenesis, at the four-cell stage, and this is reinforced by the establishment of the external protoderm in the early globular embryo, followed by further definition of internal cell layers, including the provascular tissues, by heart stage.

Protoderm formation depends at least in part on WOX function. *wox2* mutants are affected in the tangential divisions that define the protoderm at the octant stage of embryogenesis and subsequent proliferative divisions of the protoderm itself, a phenotype exacerbated by additional mutations in *WOX1* and *WOX3* or *WOX8* (Haecker *et al.*, 2004; Breuniger *et al.*, 2008). Establishment of the protoderm is associated with cell layer–specific gene expression domains, such as the *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) (Lu *et al.*, 1996), *PROTODERMAL FACTOR 2* (*PDF2*) (Abe *et al.*, 2003), and *ARABIDOPSIS CRINKLY 4* (*ACR4*, a receptor-like kinase) (Tanaka *et al.*, 2002; Gifford *et al.*, 2003) genes. *pdf2 atml1* double mutants show defective protoderm development and patterning of other embryo cells (Abe *et al.*, 2003). PDF2 and ATML1, similar to PHV and PHB, are HD-ZIP transcription factors containing a START (StAR-related lipid transfer) domain. In the RNA, this sequence can represent a target site for miRNA-mediated degradation – this occurs for the post-transcriptional processing of *PHB*, *PHV*, and *REVOLUTA* (*REV*) transcripts, as described previously, but may also represent a sterol-binding site in the protein (Lindsey *et al.*, 2003). ACR4 is also essential for root development, both in columella initial divisions and in pericycle divisions leading to the formation of lateral roots (De Smet *et al.*, 2008). Two other kinases are also required for correct development of radial pattern during embryogenesis: RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and TOADSTOOL 2 (TOAD2). Double mutants for these genes exhibit cells with incorrect identity, whereby outer cell identities are replaced by inner cell identities (Nodine *et al.*, 2007; Nodine and Tax, 2008). Vascular cell markers are expressed more widely than in wild-type (i.e., in the protoderm), whereas protodermal markers are expressed only transiently. The double mutants typically fail to develop beyond the globular stage.

MP is required for correct vascular patterning during embryogenesis, demonstrating the importance of auxin in this process, as discussed earlier (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998). Mayer *et al.* (1991) also identified the *KNOLLE* and *KEULE* genes as being essential for correct radial pattern. KNOLLE is a syntaxin-like protein (Lukowitz *et al.*, 1996; Lauber *et al.*, 1997) that physically interacts with the Sec1 protein KEULE (Assaad *et al.*, 2001), and together they regulate cytokinesis through roles in controlling vesicle fusion for correct cell plate formation. The KANADI-HD-ZIP III transcription factor system discussed previously also plays a role in radial patterning, as multiple mutants of these genes display defective establishment of the central-peripheral axis (Izhaki and Bowman, 2007).

Mutational screens have also identified regulators of the establishment of cortical and endodermal layers in the root, but which have embryonic expression – SCR and SHR (Benfey *et al.*, 1993; Scheres *et al.*, 1995; Di Laurenzio *et al.*, 1996). Loss-of-function of each gene leads to the formation of a single layer of ground tissue in the *Arabidopsis* root owing to a failure of the formative division in the cortex/endodermis initial cell, which gives rise to the two tissues. Both genes are also coexpressed in the QC of the root, and *SCR* expression is first seen in the hypophysis, before QC formation in the embryo (Wysocka-Diller *et al.*, 2000; Sabatini *et al.*, 2003). These proteins act together as a

transcription factor complex to activate a D-type cyclin required for the division of the initial cell (Sozzani *et al.*, 2010). The SHR transcription factor is mobile, moving between cell layers in the root from the site of transcription to site of protein localization in the endodermis nucleus, where it regulates cell fate (Nakajima *et al.*, 2001). It is not yet known whether it is similarly mobile in the embryo, although TMO7 is mobile between the embryo and hypophysis, as indicated earlier (Schlereth *et al.*, 2010).

Other genes required for correct embryonic radial pattern include *HYDRA1* and *FACKEL/HYDRA2*. Each of these genes is required for correct embryogenesis, with mutants showing a range of patterning abnormalities from multiple, disorganized cell layers to abnormal apical meristem function (Mayer *et al.*, 1991; Topping *et al.*, 1997; Jang *et al.*, 2000; Schrick *et al.*, 2000; Souter *et al.*, 2002). Both genes encode enzymes in sterol biosynthesis, and the mutants show defects in hormone signaling (including PIN localization) (Souter *et al.*, 2002; Pullen *et al.*, 2010), cytokinesis, and cell wall construction (Schrick *et al.*, 2004). As vascular and protoderm pattern is disrupted in these mutants, the possibility is raised that lack of specific sterols may lead to defective function of one or more START domain proteins required for radial pattern, such as ATML1, PDF2, PHV, PHB, or REV.

Conclusion and Future Directions

The development of the *Arabidopsis* embryo has provided an outstanding system in which to unravel diverse molecular mechanisms of plant development: cell division control and patterning, gene-signal interactions, cell differentiation, and morphogenesis. The precise patterning of the *Arabidopsis* embryo has greatly facilitated the genetic approach to dissecting mechanisms. We have not touched on epigenetic control in much detail, but this is surely a potentially very fruitful field for future study. Also, we do not know very much about local signalling events, such as the role of cell wall components in regulating cell identity (He *et al.*, 2007), despite some early advances in this area using *Fucus* (Souter and Lindsey, 2000).

Also on the horizon is the more extensive use of systems biology approaches, in which multiple layers of data (transcriptomics, proteomics, metabolomics, protein-protein interactions, and imaging) are integrated using advanced computational techniques to provide a more complete view of embryo development. This information will be increasingly used to inform our understanding of seed development in the major crops, essential if we are to develop a more rational, gene-based approach to improving yield and quality traits, including seedling establishment and plant architecture, to feed a growing world population.

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3 Endosperm Development

Odd-Arne Olsen and Philip W. Becraft

Introduction

The endosperm of cereal grains is an important source of food, feed, and industrial raw material. Biologically, the endosperm is considered a key factor in the evolution of land plants, helping seeds to survive under harsh conditions. The endosperm functions to nurture the embryo during early stages of development, and in species such as cereals that have persistent endosperms, it also functions as a food reserve to nourish the growing seedling during germination. These same food reserves provide nutrition to humans and livestock as well as industrial feedstock. In 1900, an unusual feature of the angiosperm seed was discovered – the embryo and the endosperm originate in two separate fertilization events, termed "double fertilization" (Nawaschin, 1898; Guignard, 1901). In this process, the embryo forms from the fusion of one haploid male gamete (sperm) and a haploid egg cell. The endosperm is derived from the fusion of a second sperm with two haploid central cell nuclei to form a triploid primary endosperm nucleus. This mode of development is called the *Polygonum* type and is typical of most familiar seeds including crops such as cereal grains and soy.

New genomics tools and resources are allowing the experimental and comparative analysis of endosperm biology at levels and scales unimaginable just a few years ago. Genome level data are rapidly accumulating on the developmental biology, molecular biology, biochemistry, physiology, and evolution of an increasing number of species. Computational tools are being developed that integrate these various levels of information to provide a systems view of endosperm function. It is hoped that such a systems-level understanding will lay the foundation for novel strategies to improve grain quality and quantity for food, feed, and industrial purposes.

Overview of Endosperm Structure and Development

An overview of the structure of the endosperm of maize, barley, rice and *Arabidopsis thaliana* is given in Figure 3.1. Cereal endosperm contains four major cell types, each with specialized functions. The starchy endosperm (central endosperm) is the major storage cell type, transfer cells transport nutrient solutes from maternal tissues into the seed, aleurone functions in mineral storage and to digest storage products to feed the germinating seedling, and the embryo surrounding cells are hypothesized to function in communication and nutrient transfer between the endosperm

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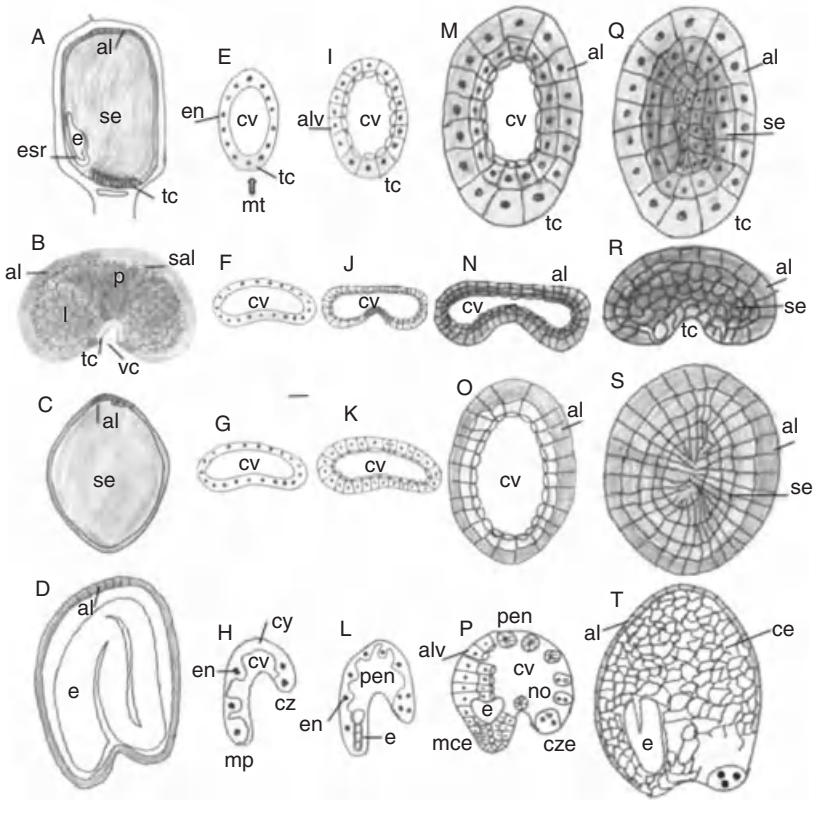

Figure 3.1 Overview of endosperm structure. *A–D*, Mature endosperm of maize *(A)*, barley *(B)*, rice *(C)*, and *Arabidopsis thaliana (D)*. al, aleurone; e, embryo; esr, embryo surrounding region; i, irregular starchy endosperm cells; p, prismatic cells; sal, subaleurone layer; se, starchy endosperm; tc, transfer cells; vc, ventral crease. *E–H*, Coenocytic stage endosperm of maize *(E)*, barley *(F)*, rice *(G)*, and *Arabidopsis thaliana (H)*. cv, central vacuole; tc, transfer cell region; mt, *Meg1* maternal transcript involved in induction of transfer cell fate specification; Mp, micropylar end; cz, chalazal end; en, endosperm nucleus; cy, endosperm cytoplasm. *I–L*, Alveolar stage of endosperm development of maize *(I)*, barley *(J)*, rice *(K)*, and *Arabidopsis thaliana (L)*; alv, alveoli; pen, peripheral endosperm. *M–P*, Cellularizing endosperm with one peripheral cell layer and inner alveoli in maize *(M)*, barley *(N)*, rice *(O)*, and *Arabidopsis thaliana (P)*. *Q–T*, Fully cellular endosperm of maize *(Q)*, barley *(R)*, rice *(S)*, and *Arabidopsis thaliana (T)*. (For color detail, see color plate section.)

and embryo. The transient endosperm of *Arabidopsis* is not a significant storage tissue, with the cotyledons of the embryo filling that role.

These and most other species of economic interest display a nuclear pattern of endosperm development, whereon nuclei proliferate in a coenocytic phase before undergoing cellularization (Brown et al. 1994; Berger, 2003; Olsen, 2004b; Sabelli and Larkins, 2009; Becraft and Gutierrez-Marcos, 2012). In contrast, cellular endosperm development lacks a coenocytic phase. Beginning at fertilization, cell proliferation involves typical cell divisions in which cytokinesis accompanies nuclear division. Although striking similarities exist in nuclear endosperm development between monocot species such as the cereals and eudicot species such as *Arabidopsis*, the nuclear mode of development is believed to have evolved independently in the two groups from a cellular ancestral state (Geeta, 2003). Consequently, it cannot be assumed that similar features of the two types of endosperms have identical genetic bases. Current phylogenetic data suggest that monocots represent a monophyletic group that shares a common ancestor, although, as shown subsequently, significant

morphological differences exist between the endosperms of the main cereals. Molecular data also support this conclusion, in that substantial portions of wheat and maize endosperm transcripts lack clear orthologues in rice (Lai *et al.*, 2004; Drea *et al.*, 2005).

At maturity, the maize endosperm (Figure 3.1*A*) consists of an internal mass of starchy endosperm cells, the major storage cell type; these cells are packed with starch granules and storage proteins. Starchy endosperm is surrounded by a single layer of aleurone cells (Becraft, 2007). Aleurone cells have thick cell walls rich in arabinoxylan (Dornez *et al.*, 2011) and a dense granular cytoplasm containing aleurone grains and other inclusions (Jones, 1969; Morrison *et al.*, 1975; Bechtel and Pomeranz, 1977). In particular, genotypes of maize, anthocyanin pigments, accumulate at maturity (Cone, 2007). Aleurone cells become desiccation tolerant and are the only living cell type in the endosperm of mature seed. When dry grains are imbibed in water, gibberellin (GA) signaling from the embryo induces the aleurone to produce and secrete hydrolases, particularly proteases and glucanases. These enzymes break down starch and storage proteins in the dead starchy endosperm cells, liberating sugars and amino acids to be absorbed by the germinating embryo. In the periphery of the starchy endosperm, one or two layers, often referred to as the subaleurone layer, contain less starch and more proteins than the central part. The basal transfer cell layer forms at the interface between the endosperm cavity and the maternal chalazal tissue. The transfer layer facilitates transfer of solutes, including amino acids, sucrose, and monosaccharides, across the plasmalemma from the maternal pedicel tissue to the endosperm compartment (Thompson *et al.*, 2001). The transfer cells also express several proteins such as BAPs (BASAL LAYER-TYPE ANTIFUNGAL PROTEINs) that have antimicrobial properties suggesting a role in defense against invading pathogens (Serna *et al.*, 2002). BETL1 is synthesized and located in basal endosperm cells, where it is tightly bound to the cell wall (Hueros *et al.*, 1995), whereas BAP2 is secreted into the intercellular matrix of the basal endosperm and accumulates predominantly in the adjacent thick-walled cell layer of the pedicel (Serna *et al.*, 2002). At early developmental stages, in the part of the starchy endosperm facing the embryo, the embryo surrounding region (ESR) forms and persists until approximately 12 days after pollination (DAP) (not shown) (Cosségal *et al.*, 2007). The function of this tissue is unknown but is hypothesized to be important for communication between the endosperm and embryo.

The barley endosperm (Figure 3.1*B*) has a similar structure, with the exception of the aleurone layer, which is three cell layers thick (Olsen and Weschke, 2012). Barley also has a subaleurone layer. In contrast to maize, the barley endosperm consists of wings with so-called irregular starchy endosperm cells and a body of prismatic starchy endosperm cells over the transfer cells toward the dorsal side. The transfer cells form in the crease of the grain and are known as modified aleurone. Although not as distinct as in maize, the barley endosperm contains cells similar to those in the ESR region. Wheat endosperm is similar in structure to barley except that the aleurone layer is typically singular but occasionally has up to three cell layers in some regions of the grain (not shown) (Drea *et al.*, 2005). In rice, the endosperm displays a radial symmetry in transverse sections giving it a tubelike appearance (Figure 3.1*C*). The aleurone layer varies in thickness from one cell layer in most regions to three cell layers. Notably, the rice endosperm lacks a distinct transfer cell layer. In mature seeds of *Arabidopsis thaliana*, the nonpersistent endosperm consists only of an aleurone layer, and the rest of the seed cavity is filled by the embryo (Vaughn and Whitehouse, 1971; Chamberlain and Horner, 1990; Groot and van Caeseele, 1993). The endosperm that initially forms after fertilization (see later) is gradually depleted as the embryo grows (Brown *et al.*, 1999).

The endosperm of maize, barley, wheat, rice, and *Arabidopsis* all have an initial coenocytic endosperm stage consisting of a thin layer of cytoplasm with nuclei lining the wall of the embryo sac (Figure 3.1*E–H*). The cereal endosperm display radial symmetries (Figure 3.1*E–G*), whereas the central cell of *Arabidopsis* has three regions that become distinct as the seed grows: the

embryo-surrounding region or micropylar endosperm (MCE), the peripheral endosperm (PEN) in the central chamber, and the chalazal endosperm (CZE) (Figure 3.1*H*) (Brown and Lemmon, 1999; Boisnard-Lorig *et al.*, 2001; Sorensen *et al.*, 2002). The coenocytic stage results from repeated nuclear divisions of the primary endosperm nucleus after fertilization without the formation of cell walls between daughter nuclei. The mechanisms for suppression of phragmoplast formation in early endosperm are unknown. The process for endosperm cellularization was first described in barley and is illustrated in Figure 3.2. A transverse section of a coenocytic stage barley endosperm and

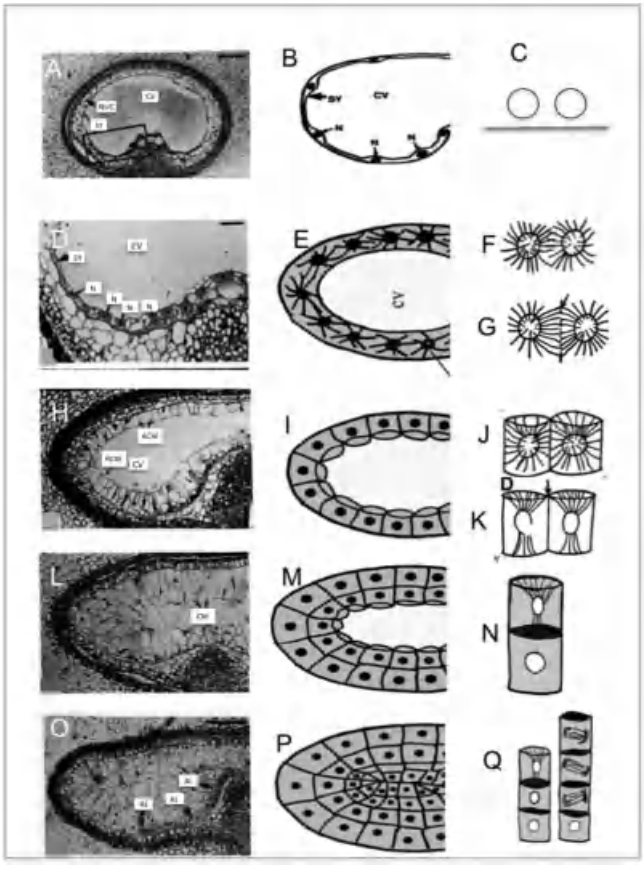

Figure 3.2 Barley endosperm cellularization process. *Left column* shows micrographs of transverse sections (2 μm) of plastic embedded barley endosperm at various developmental stages during the cellularization process. *Middle column* shows line drawings of the stage shown in the first column. *Right column* shows line drawings of endosperm nuclei and the associated microtubular arrays participating in the cellularization process. *A–C*, Coenocytic stage. Individual nuclei embedded in a thin layer of peripheral cytoplasm surround a large central vacuole. SY, coenocyte; CV, central vacuole; N, nucleus. *D–G*, Initiation of cellularization process. Radial microtubular systems (RMSs) on neighboring nuclei *(F)* form interzones acting as primitive phragmoplasts to form the alveolar cell walls *(G)*. *H–K*, Alveolar stage. Cell walls have formed alveoli around each endosperm nucleus *(J)*. A canopy of microtubules extends the alveoli toward the center of the endosperm cavity *(K)*. ACW, anticlinal cell wall; CV, central vacuole; PCW, periclinal cell wall. *L–N*, Early cellularization stage with one peripheral layer of cells and internal layer of alveoli. A periclinal division in an alveolus produces a peripheral daughter cell and an internal alveolus with its opening toward the central vacuole. *O–Q*, Fully cellular endosperm. Repetitions of the initial cellularization process in the internal alveolar layers fill in the endosperm cavity, completing the cellularization process. AL, aleurone.

line drawing representations of the same and two coenocytic nuclei are shown in Figure 3.2*A–C*. In the next phase of endosperm development, each nucleus is encased in tubelike cell wall structures (alveoli) with the openings pointing inward toward the center of the endosperm (Figure 3.2*H–K*). The formation of alveoli occurs via the so-called radial microtubular system (RMS) that forms on the membrane of coenocytic nuclei after the coenocyte has developed numerous vacuoles (Figure 3.2*D–F*). Cell walls form in the interzones between RMS from neighboring nuclei (Figure 3.2*G, arrow*). These interzones function as primitive phragmoplasts depositing the cell wall material initially consisting of a high proportion of callose. This process leads to the first layer of alveoli (Figure 3.2*H–K*). In *Arabidopsis* endosperm at the globular embryo stage, the coenocytic cytoplasm of the MCE surrounds the developing embryo, and the multinucleate PEN coenocyte is a thin peripheral layer with evenly spaced nuclei (Fig. 3.1*L*).

After the initial formation of the alveolar layer, nuclei divide periclinally with an accompanying cytokinesis, producing a peripheral layer of cells and an internal layer of alveoli (Figure 3.1*M–P*, Figure 3.2*L–N*). This process is repeated in the new alveolar layer until after several rounds the endosperm becomes fully cellular (Figure 3.1*Q–T*, Figure 3.2*O–Q*). In *Arabidopsis*, cellularization is first completed in the MCE around the embryo, whereas the PEN remains coenocytic (Figure 3.1*P*). The CZE remains coenocytic until late stages of seed maturation (Sorensen *et al.*, 2002). In contrast to cereals, the central cell mass of the *Arabidopsis* endosperm is digested during embryo growth, leaving only the peripheral layer of aleurone cells in mature seeds (Figure 3.1*D*).

Knowledge about the molecular mechanisms directing the cell cycle in coenocytic endosperm is still scarce. The key event in forming the coenocyte, phragmoplast suppression, appears similar in the species described in Figure 3.1, although in eudicot and monocot lineages, nuclear endosperm development appears to have independently evolved from the ancestral cellular mode of development (Geeta, 2003). Are the molecular controls for suppression identical? Both types of endosperm recruited the ancient program for cellularization via RMS. The basis for the halt in cell cycle progression is also unknown, as are the initiation mechanisms for the full typical plant cell cycle when the nuclei of the alveoli start to divide.

So far, little is known about the molecular biology of endosperm cellularization. As expected, the genes involved in alveolar cell wall formation are similar to the default plant mitotic cell cycle. The functions of other key genes are likely to be revealed in mutants that are arrested at the coenocytic stage. One such barley mutant, B15, suppresses phragmoplast formation to give a coenocytic endosperm that fails to cellularize (Bosnes *et al.*, 1987, 1992). Further candidates for coenocytic and cellularization genes are to be found among other barley mutants arrested at the coenocytic stage described by Ramage and Crandall (1981). Molecular cloning of barley mutant genes are now feasible as a result of the development of genomics resources, including the availability of increasingly denser genetic maps (Mayer *et al.*, 2011). Similar to barley mutants, the *ede1* mutant in *Arabidopsis* fails to cellularize, featuring an aberrant microtubule cytoskeleton where the RMS and cytokinetic phragmoplasts are absent (Pignocchi *et al.*, 2009). The EDE1 protein accumulates in nuclear caps in premitotic cells, colocalizes along microtubules of the spindle and phragmoplast, and interacts with 14-3-3 upsilon in a yeast two-hybrid assay (Pignocchi and Doonan, 2011). Another interesting approach to understanding molecular events in the coenocytic endosperm is transcriptome analysis using laser capture microdissection as reported for 4-day-old *Arabidopsis* coenocytic endosperm (Day *et al.*, 2008). These authors identified coenocytic endosperm-expressed genes that are similar to genes involved in conventional somatic mitosis with cytokinesis as well as cytoskeleton associated genes that may act to facilitate coenocytic development. Transcriptome data such as these, together with systematic mutant screens, are likely to contribute important insights into early endosperm development.

Endosperm Cell Fate Specification and Differentiation

Transfer Cells

The first molecular indication of cell fate specification reported for cereal endosperm is the differential expression of transcripts localized to the region of coenocytic endosperm above the maternal vasculature that later develops into transfer cells (modified aleurone); this implicates maternal signaling as a possible positional cue to induce transfer cell fate. One example of such a transcript is *End1* from barley (Doan et al., 1996) and wheat, which is strongly expressed in the ventral or adaxial endosperm overlying the crease at 3 DAA. The transcript continues to accumulate as the modified aleurone cellularizes and remains at a high level in the outer two cell layers through 9 DAA (Drea *et al.*, 2005). END1 is of unknown function but is similar to a family of Cys-rich proteins in *Arabidopsis* classified as proteinase inhibitors, storage proteins, and lipid transfer proteins (Drea *et al.*, 2005). In addition to *End1*, several other genes with a similar pattern of expression, including β-expansin, are detectable (Drea *et al.*, 2005).

A similar pattern of expression as for *End1* is seen in the transcript for the maize MYB-related protein-1 (MRP1, originally designated ZmMRP-1), which encodes a single Myb-repeat protein (Gomez *et al.*, 2002). This protein was shown to be necessary and sufficient to induce transfer cell differentiation in maize endosperm (Gomez *et al.*, 2009). MRP1 transcriptionally activates numerous transfer cell–specific genes in the maize endosperm (Gomez *et al.*, 2002), including *Meg1* (Gutierrez-Marcos *et al.*, 2004). Costa *et al.* (2012) showed that the *meg1* gene also acts upstream of MRP1, suggesting a positive feedback loop that establishes transfer cell fate and maintains its cellular differentiation throughout development. Strong support for this comes from decreased MRP1 expression in *meg1-RNAi* lines as well as ectopic MRP1 expression and transfer cell formation resulting from misexpression of *meg1*.

Another source of evidence for the role of maternal signaling in transfer cell development in maize comes from endosperm *in vitro* organ cultures. In such cultures, derived from isolated young endosperm at 4 DAP grown either in liquid cultures or on agar medium without maternal tissue influence, starchy endosperm and aleurone cells develop but not transfer cells (Gruis *et al.*, 2006).

Although not yet investigated, it is reasonable to speculate that transfer cell specification occurs by a similar mechanism in maize and in other cereal species with transfer cells (e.g., barley and wheat). In their survey of wheat endosperm gene expression using in situ hybridization, Drea *et al.* (2005) reported 44 transcripts that were predominant and 32 that were specific to the modified aleurone cell layer. This group included the largest number without clear orthologues in rice, a result well in line with the fact that rice lacks transfer cells.

The transfer cells are the earliest endosperm cell type to differentiate. Drea *et al.* (2005) found that the modified aleurone layer and the adjacent central endosperm region have reduced levels of histone expression by 6–7 DAA compared with the rest of the endosperm. This reduction coincides with increased calcofluor staining of cellulose demonstrating that this part of the endosperm undergoes differentiation and cell cycle exit (Drea *et al.*, 2005).

Little is known about transfer cell function in *Arabidopsis*, although the specialized pad of tissue known as the chalazal proliferating tissue positioned in the tip of the chalazal chamber has been suggested to serve a role similar to the transfer cells in cereal endosperm (Brown *et al.*, 1999). The mechanism for cell fate specification of this tissue is unknown.

The last few years have brought important insights into transfer cell developmental biology. In maize, the maternal *Meg1* transcript was identified as a long suspected maternal factor to initiate transfer cell fate specification. Strong indications that a similar system operates in barley and

wheat comes from the differential expression of the *End1* and similar transcripts in the endosperm coenocyte over the nucellar projection. In contrast, the lack of localized transfer cells in rice endosperm suggests that a similar system of *Meg1*-like and *End1*-like genes may not be present in rice. The contrast between rice and the other cereals opens up further investigations of molecular mechanisms of transfer cell specification and possibly also strategies to increase grain yield by directed changes in transfer cell function or morphology.

Starchy Endosperm Cells

Starchy endosperm cells represent the largest body of cells in the cereal endosperm (Figure 3.1*A–C*). Starchy endosperm cells accumulate starch and storage proteins. Prolamins and glutelins are encoded by transcripts that are expressed differentially in these cells (see Chapters 8 and 9). Starchy endosperm cells are derived from two sources. The first source is the inner cells of cell files that are present at the completion of endosperm cellularization (Figure 3.1*Q–S* and Figure 3.2*P*). Soon after cellularization culminates, cell division resumes in the inner cells of these files (Figure 3.2*Q*). In contrast to the alveolar divisions, which are strictly periclinal, the division planes are oriented randomly, and the cell file pattern is soon lost. The second source of starchy endosperm cells is the inner daughter cells of aleurone cells that divide periclinally giving rise to the subaleurone cells (Morrison *et al.*, 1975; Becraft *et al.*, 2000).

Cell cycle regulation in cereal endosperm has been studied most intensively in maize, where, similar to barley, a phase of mitotic cell division occurring after cellularization (Figure 3.2*Q*) is largely responsible for generating the final population of endosperm cells. This period in maize lasts 8–12 DAP in the central endosperm (Sabelli and Larkins, 2009). In maize, cell division occurs in waves, stopping first at the base of the endosperm and then in the central region. Other growth parameters, such as increases in the size of nuclei, starch granules, and cells, suggest that differentiation follows the cell division gradients (Kowles and Phillips, 1988). The mitotic index peaks around 8–10 DAP and then declines sharply. During the period from 8–é12 DAP, the maize endosperm grows rapidly to fill the seed cavity. This growth appears to be correlated with endoreduplication because the mean volume of centrally located nuclei increases roughly 10-fold (Kowles and Phillips, 1985). Endoreduplication seems to be a common feature of cereal endosperms (Chojecki *et al.*, 1986; Ramachandran and Raghavan, 1989; Giese, 1992; Kladnik *et al.*, 2006; Sabelli and Larkins, 2009). From around 9 DAP, maize starchy endosperm cells transition from a mitotic to an endoreduplication cell cycle in which repeated rounds of DNA replication without cytokinesis lead to DNA contents of as much as 96C (Sabelli and Larkins, 2009). Similar to animals, plant cell cycle progression is regulated by cyclin-dependent kinases (CDKs) and their cyclin partners, CDK inhibitors, and retinoblastoma-related (RBR) proteins (Sabelli and Larkins, 2009). In maize endosperm, CDK activity peaks at the time of the onset of endoreduplication (10–12 DAP), convincingly supporting the view that the switch from the mitotic to the endoreduplication cell cycle occurs via downregulation of mitotic CDKs and upregulation of S-phase CDKs (Grafi and Larkins, 1995; Sun *et al.*, 1999; Leiva-Neto *et al.*, 2004; Coelho *et al.*, 2005; Sabelli and Larkins, 2009). Increasing evidence also implicates RBRs in endosperm development (Sabelli and Larkins, 2009). The function of endosperm endoreduplication remains unresolved because impairing endoreduplication had no obvious effect on grain size or yield (Sabelli and Larkins, 2009).

In their analysis of wheat endosperm transcription, Drea *et al.* (2005) detected 26 transcripts that were central endosperm predominant and 46 that were central endosperm specific. Among transcripts in the first group, most were shared between starchy endosperm, transfer cells, and

aleurone (e.g., the formate dehydrogenase transcript, which was expressed in all cell types until at least 13 DAA). Other transcripts continued expressing in the starchy endosperm while being downregulated in the aleurone (e.g., α-thionin in a pattern similar to the storage protein gliadin that starts to accumulate in the starchy endosperm at 9 DAA). Among the investigated wheat starchy endosperm transcripts, the cereal with the largest divergence was rice.

Around the midpoint of seed development, cereal endosperms undergo programmed cell death (PCD), which was suggested by Nguyen *et al.* (2007) to facilitate nutrient hydrolysis and uptake by the embryo at germination. PCD in maize starchy endosperm initiates at approximately 16 DAP in the central starchy endosperm cells and in apical cells near the silk scar and eventually leads to the death of the half-top of the endosperm at 28 DAP (Young and Gallie, 1999). In wheat, PCD initiates stochastically and leads to the death of all starchy endosperm cells by 30 DAP (Young and Gallie, 1999). PCD in plants bears typical characteristics of some PCD in animals, including DNA fragmentation, but the effector caspases have not been clearly identified. Instead, evidence points to a role of proteases with caspase-like activity (Hatsugai *et al.*, 2004; Nguyen *et al.*, 2007). Endosperm PCD is novel compared with typical PCD programs where cellular corpse processing occurs concurrently with cell death and is controlled by an endogenous cellular program. In endosperm by contrast, cellular corpse processing is temporally delayed until germination and is controlled exogenously by the activities of aleurone cells (van Doorn *et al.*, 2011).

Ethylene is a clear candidate as a regulator of PCD (Young *et al.*, 1997). In addition, ABA biosynthesis affects PCD indirectly, via ethylene biosynthesis, as shown by increased ethylene levels and PCD in maize viviparous mutants in which the ABA biosynthetic pathway is compromised (Young *et al.*, 1997; Sabelli and Larkins, 2009; Becraft and Gutierrez-Marcos, 2012).

Aleurone Cells

Cell fate specification of aleurone cells in maize follows a simple pattern described by the so-called surface rule; all cells positioned on the surface of the endosperm assume aleurone cell fate, and interior cells become starchy endosperm cells (Olsen, 2004a). An illustration of this principle is easily observable with *in vitro* maize endosperm cultures in which aleurone cells and starchy endosperm cells are marked with cell-specific green and cyan fluorescent proteins (Figure 3.3) (Gruis *et al.*, 2006). In such cultures, the endosperm becomes fragmented and irregular. Regardless

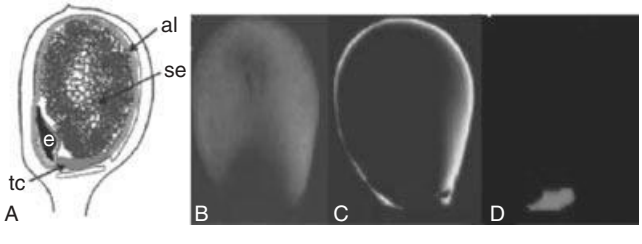

Figure 3.3 Kernel of "triple marked" maize endosperm. *A*, Diagram of the three major endosperm tissues. al, aleurone; e, embryo; se, starchy endosperm; tc, transfer cells. *B–D*, Expression of fluorescent cell-type markers in 12 DAP endosperm. Various cell-specific promoters are used to regulate expression of fluorescent proteins with differing spectral properties.B, γ-Zein:AmCyan expression fluoresces blue in the starchy endosperm. *C*, Ltp2:ZsYellow expression is specific for the aleurone layer. *D*, End1:DsRed expression marks transfer cells. (Modified from Gruis *et al.* [2006].) (For color detail, see color plate section.)

of shape, all surfaces are covered by aleurone cells (green); internal cells have starchy endosperm identity and fluoresce blue.

The fate of aleurone cells appears to be specified early in endosperm development. As described previously, the surface layer of endosperm displays a distinct behavior from the internal cells at the outset of cellularization, suggesting that independent developmental trajectories for aleurone and starchy endosperm are already established. Transcriptomic analysis in wheat further shows that by 6 DAA the surface layer has acquired a molecular signature of aleurone, very different from starchy endosperm (Gillies *et al.*, 2012). Although aleurone cells have not fully differentiated by this point, the transcriptional profile is more similar to later stage aleurone than to internal cells.

Despite the early establishment of distinct position-dependent cell identities, cell fate in the endosperm periphery remains flexible throughout endosperm development. In culture, starchy endosperm cells that become positioned on the surface transdifferentiate to aleurone cells, and aleurone cells that become internalized convert to starchy endosperm (Gruis *et al.*, 2006; Reyes *et al.*, 2010, 2011). Also *in planta*, aleurone cell fate remains plastic throughout late stages of endosperm development, up to the last cell division, even after they have acquired the typical aleurone cytological features (Becraft and Asuncion-Crabb, 2000; Becraft and Yi, 2011). A signaling system thus functions throughout endosperm development to specify and maintain aleurone cell identity.

Genetic studies in maize have identified several components of a signaling system required for aleurone cell differentiation. Mutations in the *crinkly4* (*cr4*) and *defective kernel1* (*dek1*) genes disrupt aleurone cell fate specification indicating these genes are positively acting factors required for this process (Becraft *et al.*, 1996, 2002; Lid *et al.*, 2002). CR4 is a receptor kinase (Becraft *et al.*, 1996; Jin *et al.*, 2000), and DEK1 is a novel plasmamembrane–localized protein with 21 transmembrane helices, an extracellular loop domain, and a cytoplasmic domain containing a calpain-like protease (Lid *et al.*, 2002; Wang *et al.*, 2003). Genetic studies suggest CR4 and DEK1 function together in a signaling system (Becraft *et al.*, 2002), and the colocalization of these proteins in endocytic vesicles supports that hypothesis (Tian *et al.*, 2007). Negatively acting factors also have been identified in maize. The *supernumerary aleurone layers1* (*sal1*) mutant produces multiple aleurone layers instead of the typical single layer (Shen *et al.*, 2003). SAL1 has homology to class E vacuolar sorting proteins, suggesting that it might regulate aleurone cell number by functioning in retrograde cycling of the DEK1 or CR4 proteins from the plasmamembrane. This would serve to dampen the amplitude of signaling and restrict aleurone cell fate to just the outer layer. In the *sal1* mutant, DEK1 or CR4, or both, would accumulate and provide a higher level of signaling to promote aleurone differentiation. The colocalization of SAL1 with DEK1 and CR4 in endocytic vesicles is consistent with this hypothesis (Tian *et al.*, 2007). This would place SAL1 upstream of DEK1 and CR4 in the regulatory pathway. The *thk1* mutant also causes multiple aleurone layers; however, this mutant is epistatic to *dek1*, suggesting that THK1 functions downstream of DEK1 (Yi *et al.*, 2011). The molecular identity of THK1 is unknown. Other factors have been identified in rice and barley. A double knockdown of RISBZ1 and RPBF transcription factors caused a multilayered, disordered aleurone in rice (Kawakatsu *et al.*, 2009). How or if these functions relate to the DEK1 signaling system is unknown. The *defective seed1* (*des5/B5*) mutant of barley creates a single aleurone layer, as opposed to the normal three layers, suggesting the normal gene functions to promote multilayering (Olsen *et al.*, 2008). *HvCr4* transcript levels were significantly decreased in *des5*, consistent with the hypothesis that the level of CR4 signaling might modulate aleurone cell layer number. However, the inheritance of aleurone layer number as a quantitative trait argues that the regulation could be complex and involve additional factors yet to be discovered (Jestin *et al.*, 2008).

There have also been indications of hormonal functions in aleurone specification. Expression of isopentenyl transferase under the control of a senescence inducible SAG-12 promoter induced aleurone mosaicism in the crowns of maize kernels suggesting that cytokinin can inhibit aleurone differentiation (Geisler-Lee and Gallie, 2005). Treating plants with the auxin transport inhibitor NPA caused a multiple aleurone cell layer suggesting that auxins also function in regulating aleurone fate (Forestan *et al.*, 2010).

Mutant analyses have also identified a collection of genes that appear to function downstream of the cell fate decision and to be required for aleurone differentiation (Gavazzi *et al.*, 1997; Becraft and Asuncion-Crabb, 2000; Lid *et al.*, 2004). As of yet, the molecular identification of these genes have not been reported.

At late stages of development, during seed maturation, the aleurone cells are the only endosperm cells not to undergo cell death. The aleurone layer undergoes ABA-dependent maturation, which involves the expression of dehydrins and the acquisition of desiccation tolerance, similar to the embryo maturation program. The VIVIPAROUS1 (VP1) transcription factor and ABA biosynthetic enzymes are required for this process. In viviparous mutants, the aleurone layer prematurely enters the germination program and begins secreting hydrolytic enzymes to digest the contents of starchy endosperm cells.

In contrast to the starchy endosperm, programmed cell death occurs during germination in aleurone cells and is induced by GA, not ethylene (Nguyen *et al.*, 2007). A transcriptome analysis of barley showed that proteases and the ethylene and ABA pathways are implicated in endosperm PCD and maturation during barley grain development (Sreenivasulu *et al.*, 2008).

In summary, aleurone cell fate specification in maize endosperm follows a simple rule; cells on the surface become aleurone cells, whereas interior cells are specified as starchy endosperm cells. The *Dek1* and *Cr4* genes are known to act in aleurone cell specification. The molecular basis for three aleurone cell layers in barley, instead of one layer as in maize and wheat, is currently unknown. Cloning of the mutant genes that give multiple layers in maize and a single layer in barley may reveal the mechanism regulating aleurone cell layer numbers in cereals, opening up strategies to increase the number of oil-containing, nutritious aleurone cells in species with only a single cell layer.

Embryo-Surrounding Region

The embryo-surrounding region (ESR) has been most intensively studied in maize where it lines the cavity of the endosperm in which the embryo develops. These cells have dense cytoplasmic contents (Schel *et al.*, 1984; Schel and Kieft, 1986; Kowles and Phillips, 1988) and are characterized by the expression of the *Esr1*, *Esr2*, *Esr3* (Opsahl-Ferstad *et al.*, 1997), *ZmAE1* (*Zea mays androgenic embryo1*), and *ZmAE3* (Magnard *et al.*, 2000) genes between 5 and 20 DAP. The ESR3 protein is part of a family of small hydrophilic proteins that share a conserved motif with CLAVATA3 (CLV3), a ligand of the receptor-like kinases CLV1 and CLV2 functioning to regulate meristem size in *Arabidopsis* (Fletcher *et al.*, 1999; Ogawa *et al.*, 2008).

Future studies are needed to clarify the role of ESR proteins in ESR signaling. The function of the ESR in maize is unknown but may include a role in embryo nutrition or in communication between the embryo and endosperm, or both. The ESR cells disintegrate and disappear at 12 DAP (Kieselbach and Walker, 1952). Barley lacks a prominent ESR region, but a small part of the endosperm situated close to the embryo that appears to be similar to the maize ESR was termed “zone 1” by Engell (1989).

Genomic Resources

Among the cereals, the most extensive genomic resources are available for rice and maize. Each has a sequenced genome, microarrays, genetic transformation, and sequence indexed insertional mutant collections (Settles *et al.*, 2007; Jung *et al.*, 2008; Vollbrecht *et al.*, 2010). In addition, other resources such as fluorescent protein markers to identify various cell types or subcellular compartments are being developed (Mohanty *et al.*, 2009). Such markers greatly facilitate developmental and genetic studies as exemplified by the work of Gruis *et al.* (2006). These investigators used a "triple marked" line containing transgenes specifically labeling aleurone, starchy endosperm, and transfer cells (Figure 3.3) and demonstrated the ability of *in vitro* cultured endosperm to differentiate aleurone and starchy endosperm but not transfer cells. Figure 3.4 illustrates the use of such markers to analyze the effects of mutations on the differentiation of various cell types. Among the small grain cereals, wheat genome sequencing is currently underway, and a physical map for barley has been published, both using single chromosome arm isolation, BAC library construction, and sequencing

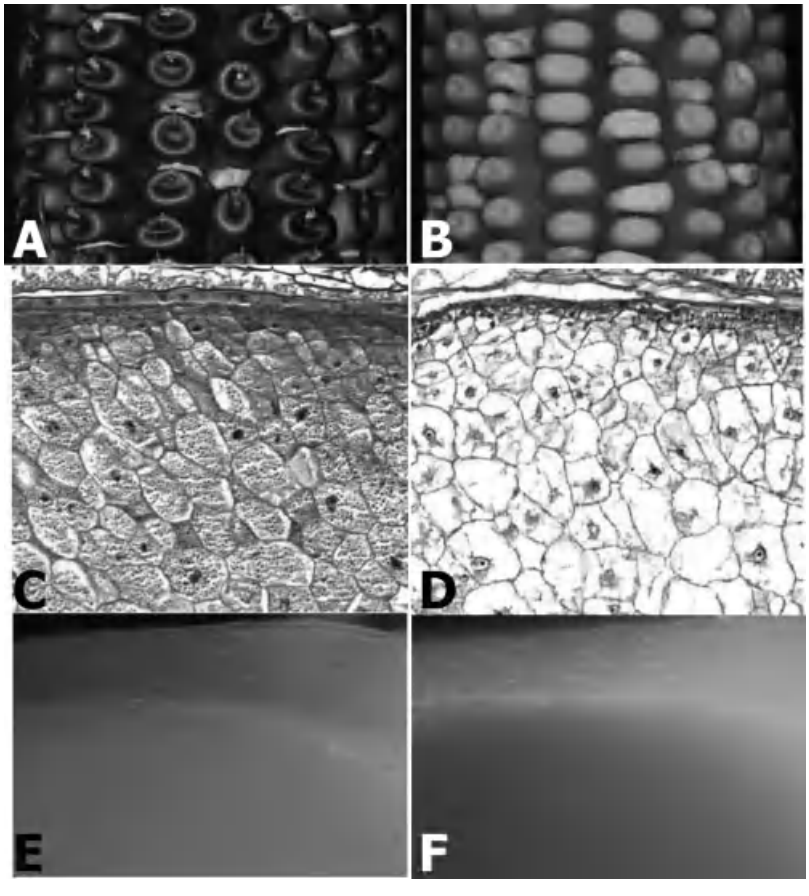

Figure 3.4 Examples of maize kernel mutants. *A*, Ear segregating an *emp* mutant. *B*, Ear segregating a *dek* mutant. *C*, Histological section of a wild-type kernel with cells packed with starch grains. *D*, Section of a mutant endosperm defective in starch accumulation. *E*, Expression of an FL2-RFP fluorescent marker for α-zein accumulation in wild-type (Mohanty *et al.*, 2009). *F*, Lack of FL2-RFP expression in a mutant kernel. (For color detail, see color plate section.)

of individual chromosome arms (Mayer *et al.*, 2011). These technological advances are expected to faciltitate the identification of mutant genes in wheat and barley in the near future.

Maize has particularly rich collections of seed mutants, many of which are available through the Maize Genetics Cooperation Stock Center (Sachs, 2009). The first systematic collection of Mu-tagged maize lines for reverse genetics screening, TUSC (Trait Utility System for Corn), facilitated the cloning of several maize genes, including *An1* (Bensen *et al.*,1995), *Dek1*, and *Sal1* (Table 3.1) (Lid *et al.*, 2002; Shen *et al.*, 2003). A large-scale mutagenesis with *Mutator* transposons produced a collection of nearly 2000 independent maize mutants with kernel phenotypes (McCarty *et al.*, 2005; Settles *et al.*, 2007). More than half of these mutants showed mutant phenotypes known as *empty pericarp* (*emp*) or *defective kernel* (*dek*) (Figure 3.4), depending on the severity of the phenotype; more filial tissue is apparent in *dek* than *emp* mutant kernels (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980; Scanlon *et al.*, 1994). There is overlap in *emp* and *dek* mutant classes and some mutants with variable expressivity often display both phenotypes. Genes producing *emp* or *dek* mutant phenotypes are essential for endosperm or endosperm and embryo development. Genes required specifically for embryo development produce embryo-specific (*emb*) mutant phenotypes (Clark and Sheridan, 1991; Sheridan and Clark, 1993). Although *emb* mutants produce nearly normal endosperm, *emp* and *dek* mutants produce neither normal endosperm nor embryos. Embryos can be rescued in culture for some *dek* mutants (Sheridan and Neuffer, 1980), demonstrating that embryo viability is dependent on endosperm functions but not vice versa.

An early and conservative estimate suggested there are >285 loci essential for maize seed viability, based on allele frequencies in EMS-induced seed mutants (Neuffer and Sheridan, 1980). Subsequent analyses identified multiple essential genes not identified in the earlier study, verifying that the estimate is low (Clark and Sheridan, 1991; Scanlon *et al.*, 1994; Costa *et al.*, 2003; Gutierrez-Marcos *et al.*, 2006; Yi *et al.*, 2009). *Arabidopsis* is estimated to have 500–750 genes essential for seed development (see Chapter 1) (McElver *et al.*, 2001). Analysis of >300 such genes revealed that virtually every class of cellular function was represented (McElver *et al.*, 2001; Tzafrir *et al.*, 2004; Drea *et al.*, 2005; Devic, 2008; Meinke *et al.*, 2008). A unifying theme was that genes essential for seed development are less likely than other genes to contain paralogs in the genome.

Available information on the molecular identity of seed-lethal mutants in maize (Table 3.1) is consistent with the array of functions seen in *Arabidopsis*, although the data are currently too sparse for general conclusions. The large number, indistinct macroscopic phenotypes, and lethality of *dek/emp* mutants make them difficult to study using traditional approaches. Although essential genes are likely to be involved in all aspects of seed biology, including development, this important class of mutants has been poorly studied. These mutants, including use of the high-throughput maize *in vitro* endosperm culture system for transformation of aleurone and starchy endosperm cells (Reyes *et al.*, 2010) are promising tools for enhancing understanding of cereal endosperm development.

Transcriptional Profiling of Endosperm Development

Transcriptomic analyses have been performed for most of the major grain cereals, including maize, rice, wheat, and barley (Laudencia-Chingcuanco *et al.*, 2007; Hansen *et al.*, 2009; Liu *et al.*, 2010; Sekhon *et al.*, 2011; Gillies *et al.*, 2012; Xue *et al.*, 2012). As would be expected for cereals, genes involved in sucrose and starch metabolism and protein synthesis were enriched in the endosperm. Additionally, genes involved in defense, signal transduction, and transcription are highly represented.

Table 3.1 Known Maize *dek/emp* Mutant Genes

Mutant	Gene ID	Molecular Function	References
defective kernel1 (dek1)	GRMZM2G321753	21-transmembrane calpain protease	Lid *et al.*, 2002; Wang *et al.*, 2003
Supernumerary aleurone layer 1 (sal1)	GRMZM2G117935	Endosome trafficking	Shen *et al.*, 2003
opaque5 (o5)	GRMZM2G142873	Monogalactosyldiacylglycerol synthase	Myers *et al.*, 2011
discolored1 (dsc1)	GRMZM2G117329	ADP–ribosylation factor–GTPase activating protein	Takacs *et al.*, 2012
empty pericarp2 (emp2)	GRMZM2G039155	Heat shock factor binding protein	Fu *et al.*, 2002
empty pericarp4 (emp4)	GRMZM2G092198	Mitochondrion-targeted pentatricopeptide repeat protein	Gutierrez-Marcos *et al.*, 2007
indeterminate gametophyte1 (ig1)	GRMZM2G118250	LOB transcription factor	Evans, 2007
zmk1	GRMZM2G171279 (ambiguous – could be paralog)	Auxin-induced K^+ uptake channel	Philippar *et al.*, 2006
etched1 (et1)	GRMZM2G157574	Plastidal transcription factor	da Costa e Silva *et al.*, 2004

In rice, transcripts from 18,401 of the 46,857 gene models were expressed in developing endosperm, analyzed at 3, 6, 9, and 16 DAP. Hence, slightly more than a third of the genome is involved in endosperm development and function (Xue *et al.*, 2012). Given the unique aspects of endosperm function and development, surprisingly few genes are endosperm-specific. Microarray analysis of developing rice caryopses identified 474 genes expressed in endosperm but not embryo (Xue *et al.*, 2012). Microarray analysis during a time course of maize seed development identified 168 endosperm-specific genes (Sekhon *et al.*, 2011). In contrast, deep sequencing of maize transcriptomes identified no genes specific to the 14 DAP endosperm, although embryo-specific genes were found (Waters *et al.*, 2011). The contrasting results likely reflect the different sensitivities of deep sequencing versus microarray methods. Additionally, the number of genes expressed in 16 DAP rice endosperm was significantly less than at earlier stages (Xue *et al.*, 2012), suggesting that a fuller examination of different developmental stages and more precise dissection of specific regions or cell types would likely reveal genes expressed uniquely in the endosperm. Consistent with this, Gillies *et al.* (2012) reported in wheat that the transcriptomes of the starchy endosperm and aleurone were substantially different from one another and that both changed dynamically during development. Nonetheless, the paucity of endosperm-specific genes is a surprising result given the unique and highly specialized aspects of endosperm development and function.

A coexpression meta-analysis of rice microarray data suggested that endosperm-specific transcription factors were largely involved in nutrient storage and response to abscisic acid (Xue *et al.*, 2012). Numerous protein kinase genes were found to be predominantly expressed in the endosperm, including two genes for SnRKs, involved in regulating carbohydrate metabolism, and many receptor-like kinases, calcium-dependent kinases, and casein kinases. These were suggested to function in combination with the transcription factors to regulate cellular and metabolic networks involved with endosperm development (Xue *et al.*, 2012).

In a time course of maize grain development, principle component analysis of global gene expression showed that whole seeds from 2–8 DAP clustered together, possibly reflecting high mitotic activity in the endosperm during that period (Sekhon *et al.*, 2011). Transcription profiles in the endosperm underwent a transition from 12–20 DAP, after which little change occurred up through 24 DAP. At this point, the endosperm reaches a steady state where cell division and differentiation occur around the periphery of the endosperm, with a gradient of storage product accumulation and cell maturation progressing inward until finally internal cells undergo PCD. This steady state would be maintained until grains underwent desiccation, which would likely involve expression of new genes. Similar transitions were observed in barley (Hansen *et al.*, 2009), and reprogramming occurred in wheat between 3 and 7 DAA, between 7 and 14 DAA, and between 21 and 28 DAA (Laudencia-Chingcuanco *et al.*, 2007). These periods correspond to endosperm cellularization and proliferation, the onset of grain filling, and seed maturation and desiccation.

Gillies *et al.* (2012) similarly found a dynamic profile with RNAseq analysis. They analyzed wheat aleurone and starchy endosperm at 6, 9, and 14 DAA. Gene expression differences reflected the different metabolic activities of the two cell types (e.g., lipid versus starch accumulation). Less predictable were differences in classes of regulatory activities, such as a higher level of receptor signaling functions in aleurone. Also, the two tissues appeared to be under differential stress that changed over time. Although both tissues showed dynamic expression profiles as expected for a developmental progression, the major changes occurred at different times. In starchy endosperm, there was a major reprogramming between 6 and 9 DAA as the tissue transitioned from proliferation to grain filling, whereas in aleurone the major changes occurred at 14 DAA as the cells become fully differentiated. These findings reinforce the need to examine subpopulations of endosperm cells over a time course to understand fully the developmental progression of cellular activities.

Considering the high levels of conservation among genomes of cereals and in the functions of cereal endosperms, there appears to be surprising diversity in endosperm transcriptomes. In a comparative study of maize and rice, 78% of maize endosperm cDNAs mapped to homologous sequences of the rice genome, leaving 22% of the endosperm transcriptome unique to maize (Lai *et al.*, 2004); 4139 unique maize cDNAs mapped to 3108 rice loci suggesting that many of these genes were tandemly duplicated in rice. This highlights two reasons why genomic analyses must be performed for each individual species: (1) not all genes are present in every species and can be studied only where they reside; (2) because duplicated genes are less likely to show mutant phenotypes, the array of genes amenable to mutant analysis likely differs substantially among cereal species.

Gene Imprinting in Cereal Endosperm

Gene imprinting refers to the differential expression of a gene depending on whether it was inherited from the female or male parent. Imprinting occurs in plants and mammals and is most prevalent in extraembryonic tissues that provide nutritional support to the developing embryo: the placenta in mammals and the endosperm in plants. The function of imprinting is generally believed to relate to controlling resource allocation among offspring (Haig, 2004). Endosperm gene imprinting has been extensively studied in *Arabidopsis* and is discussed in detail in Chapter 4. In cereals, more recent global transcript profiling studies using deep sequencing approaches on seeds from reciprocal crosses have begun to shed additional light on the extent of imprinting, have confirmed the prevalence of imprinting in the endosperm compared with embryos, and have provided new insights into mechanisms and the potential functional significance of imprinting. Such analyses

identified 165 maternally and 43 paternally expressed genes in *Arabidopsis* endosperm (Gehring *et al.*, 2011), 93 maternally and 72 paternally biased genes in rice (Luo *et al.*, 2011), and 68 maternally and 111 paternally preferred genes in maize (Zhang *et al.*, 2011). Imprinted genes appear to be enriched for regulatory functions, including nucleic acid binding factors, kinases, and chromatin regulatory factors (Waters *et al.*, 2011). Additionally, imprinted noncoding transcripts were identified in rice and maize (Luo *et al.*, 2011; Zhang *et al.*, 2011). In maize, several paternally expressed noncoding RNAs mapped to intronic regions of maternal-specific protein-coding genes (Zhang *et al.*, 2011), reminiscent of an imprinting mechanism known in mammals (Frost and Moore, 2010). Other observations hint at previously unknown imprinting mechanisms. DNA methylation was examined in maize, and all imprinted loci showed maternal hypomethylation, regardless of whether the gene was maternally or paternally expressed (Waters *et al.*, 2011; Zhang *et al.*, 2011). Such genes are likely subject to regulation by a polycomb group repressive complex, as described in Chapter 4. In rice, parent-specific alternative transcript splicing was also observed (Luo *et al.*, 2011). Among the functional classes of imprinted genes were a prevalence of genes involved in chromatin modeling and regulatory genes such as signal transduction or transcription factors (Gehring *et al.*, 2011; Zhang *et al.*, 2011).

The function of imprinting is generally hypothesized to regulate nutrient allocation among offspring, as has been demonstrated in mammals. Functional studies lend strong support to this hypothesis. The *meg1* gene, described earlier, is important for transfer cell differentiation and is an imprinted gene with only the maternal allele expressed in the early endosperm. Replacing the promoter with a nonimprinted transfer cell promoter resulted in more extensive transfer layer formation and an increase in seed size (Costa *et al.*, 2012). Imprinting the male copy serves to place nutrient allocation under maternal control.

Several lines of evidence suggest that nutrient allocation might not be the only purpose for imprinting. A generally low level of conservation in imprinted genes among *Arabidopsis*, rice, and maize (Gehring *et al.*, 2011; Hsieh *et al.*, 2011; Luo *et al.*, 2011; Zhang *et al.*, 2011) could be an argument that most imprinting is unlikely to control genes of fundamental importance for seed development. However, low conservation levels among imprinted placental genes are observed between mouse and human where important developmental functions are well established (Frost and Moore, 2010), and a limited overlap was observed among different *Arabidopsis* datasets (Gehring *et al.*, 2011; Hsieh *et al.*, 2011), so these comparisons must be viewed with caution. There are some examples of conserved imprinted genes among maize, rice, and *Arabidopsis* (Waters *et al.*, 2011; Zhang *et al.*, 2011). However, the facts that imprinting in some genes is allele-specific (Kermicle, 1970; Waters *et al.*, 2011; Zhang *et al.*, 2011) and that transposon insertions are often associated with imprinted loci (Wolff *et al.*, 2011) suggest that some imprinting may be a manifestation of transposon silencing mechanisms that maintain genome integrity during reproductive development (Wollmann and Berger, 2011).

Conclusion

Endosperm development displays several features that are novel relative to the rest of plant development. Early stages of endosperm development are highly conserved among plant species, whereas later stages differ substantially. Cereal endosperm has received particular attention because of its persistence in mature grains and because of its significance for food, feed, and industrial uses. Genomic analyses have validated the high degree of genetic conservation among cereal species but have also highlighted differences. Although much information will translate among cereal

species, a substantial amount will not, and analyses must be conducted for each system. New genomic tools and resources being rapidly developed for functional and comparative studies will quickly increase our understanding of endosperm development to a high level of sophistication. With that, our ability to improve and engineer endosperm properties for human needs will be greatly enhanced.

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4 Epigenetic Control of Seed Gene Imprinting

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Introduction

Genomic imprinting is a process found in mammals and flowering plants and is defined as the monoallelic expression of a gene in a parent-of-origin dependent manner. Imprinting is established epigenetically because the two alleles of an imprinted gene are identical in DNA sequence but differ in expression state. Epigenetic marks, mainly DNA methylation and histone modifications, are often associated with repressed chromatin and reside on specific parental alleles resulting in transcriptional silencing (Allis *et al.*, 2007). In both plants and mammals, most genomic imprints are established after meiosis in the gametes and, for the most part, remain after fertilization. To date, the number of imprinted genes in mammals is ∼120, representing <1% of the genes in the genome (Glaser *et al.*, 2006).

In the case of the flowering plant *Arabidopsis thaliana*, the current number of imprinted genes is ∼50, a finding resulting from several more recent high-throughput allele-specific transcriptome studies (Tiwari *et al.*, 2010; Gehring *et al.*, 2011; Hsieh *et al.*, 2011; McKeown *et al.*, 2011; Wolff *et al.*, 2011). Similar genome-wide imprinting screens in maize (Waters *et al.*, 2011) and rice (Luo *et al.*, 2011) have also greatly increased the number of known imprinted genes in these agriculturally significant monocots. As described subsequently, the plant-imprinting field has graduated to the "omics" era, as a considerable number of transcriptome, methylome, and epigenome studies have appeared in the literature in the last few years.

Genomic Imprinting and Parental Conflict Theory

Parental Conflict Theory

Genomic imprinting is a process that results in only a single allele of a gene being expressed. As such, it seems a precarious evolutionary choice, reducing the robustness of the genome and potentially reducing the fitness of the organism. This is especially so in the case of imprinted genes, which have been shown to be indispensible for mammalian and plant development (Huh *et al.*, 2008; Bartolomei and Ferguson-Smith, 2011; Wilkins and Ubeda, 2011).

Several theories have been proposed attempting to explain the evolutionary origins of genomic imprinting (Haig, 2000; Day and Bonduriansky, 2004; Wolf and Hager, 2006). However, the most

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widely accepted theory is the kinship or parental conflict theory (Haig, 2000). This theory suggests that imprinting arose from a conflict between parents regarding how much of the mother's nutrients should support her offspring from potentially multiple fathers (Moore and Haig, 1991). From an evolutionary perspective, there is no drive for the father to favor maternal nutrient distribution to the offspring of other broods. His main aim is to promote the growth of the fetus and the placenta, possibly at the cost of other fetuses, which may not be his, both in the current and in future pregnancies, maximizing the propagation of his genes. Conversely, the mother wishes to curb the growth of the fetus and the placenta, preserving her fecundity and the viability of other fetuses she may be carrying or wishes to carry in the future, maximizing the propagation of her genes.

This conflict is potentially powerful enough to promote the evolution of unequal gene expression between selected parental alleles over time. Genes promoting the increased allocation of maternal resources to the offspring would be expressed from the paternally inherited genome (paternally expressed genes [PEGs]) and repressed on the maternally inherited genome. Genes promoting a more equal distribution of maternal resources to the offspring would be expressed on the maternally inherited genome (maternally expressed genes [MEGs]) and repressed on the paternally inherited genome.

Parental Conflict Occurs in Tissues That Support the Embryo

The mammalian placenta and flowering plant endosperm are similar in function, being directly responsible for bringing maternal and embryonic circulation into contact, facilitating nutrient exchange and determining resource allocation to the embryo. They both serve as important foci for potential parental conflict (Frost and Moore, 2010; Li and Dickinson, 2010). The evolution of the placenta has been significantly advantageous to mammals, providing the fetus with a longer gestation period resulting in a more developed offspring at birth (Constancia *et al.*, 2004). Of the ~120 imprinted genes currently known in mammals, most are expressed in both the fetus and the placenta. A subset of imprinted genes are expressed only in the placenta, and some genes are more widely expressed but imprinted only in the placenta and not the fetus (Lewis *et al.*, 2004; Coan *et al.*, 2005; Monk *et al.*, 2009). The ontology of many mammalian imprinted genes shows them to be important in regulating the placenta's ability to transfer nutrients and to regulate embryo development (Constancia *et al.*, 2004; Gregg *et al.*, 2010).

The flowering plant endosperm acts as a conduit between the sporophyte and the embryo, working to transfer maternal nutrients to the embryo. In addition, the endosperm has been shown to synthesize extensive amounts of lipids, proteins, and starch (Olsen, 2004; Huh *et al.*, 2008). In dicots, such as *Arabidopsis*, the endosperm is mostly consumed during seed development by the embryo, whereas in monocots it persists in the mature seed and is consumed by the embryo during germination (Olsen, 2004; Brown and Lemmon, 2007). Persistent endosperm provides a selective advantage for monocots (i.e., cereals) allowing for better seed dispersal, long-term dormancy, and effective seedling establishment.

The endosperm has long been recognized for its nutritive value. The cereal endosperm has been highly advantageous to humans, being the primary source of nutrition worldwide, providing at least ≥60% of daily caloric intake (http://www.who.int/nutrition/topics/3_foodconsumption/en/index1.html). Almost all genomic imprinting in plants occurs solely in the endosperm, a situation reminiscent of mammals, where the placenta is the focal organ for imprinting. This highlights the potential importance of the flowering plant endosperm as a tissue that promotes parental conflict.

Parental Conflict Predicts That Imprinted Genes Will Have Embryo-Supporting Functions

In mammals, the physiology of several imprinted genes is strongly supportive of the parental conflict theory. A key example is *insulin-like growth factor 2 (Igf2)*, a highly potent growth factor that is paternally expressed in the mammalian fetus and placenta. In mice, a reduction in *Igf2* expression leads to a 40% reduction in fetal growth (DeChiara *et al.*, 1990). Manipulation of the *Igf2/H19* imprinting control region to promote biallelic *Igf2* transcription results in fetal and placental overgrowth (Leighton *et al.*, 1995), a molecular etiology and phenotype mirrored by Beckwith-Wiedemann syndrome in humans (Cooper *et al.*, 2005). This overgrowth is also true globally, demonstrated by the phenotypes resulting from the creation of uniparental mice. Concepti carrying paternal-only genetic material consist of vestigial embryonic material, with overgrowth of placental trophoblast cells, and concepti with maternal-only genetic material consist of a disorganized embryo, with little or no placental material (McGrath and Solter, 1984; Surani *et al.*, 1984).

Some imprinted genes found in the *Arabidopsis* endosperm appear also to have functions in accordance with the parental conflict theory. Two of the best examples are the well-studied MEGs *MEDEA* (*MEA*) (Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999) and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) (Luo *et al.*, 1999). *MEA* encodes as SET-domain protein that is homologous to *Drosophila Enhancer of Zeste [E(z)]*, whereas *FIS2* encodes a zinc-finger transcription factor homologous to *Drosophila Suppressor of Zeste 12 [Su(z)12]* (Grossniklaus *et al.*, 1998; Luo *et al.*, 1999). SET domains in *Arabidopsis* are responsible for the post-translational methylation of lysine residues on histone tails. In *Drosophila*, both E(z) and Su(z)12 along with other proteins form the polycomb group (PcG) complex, which carries out transcriptional gene silencing through trimethylation (me3) of the 27th lysine residue on the tail of histone H3 (H3K27). Remarkably, loss-of-function mutations in both *MEA* and *FIS2* produce autonomous endosperm without ever undergoing fertilization. This endosperm is severely compromised as a consequence of failing to undergo the distinct phases of endosperm development (i.e., nuclear proliferation, cellularization, and maturation) and is marked by excessive nuclear proliferation, a loss of anterior and posterior polarity, and an aborted embryo. Both *mea* and *fis2* phenotypes suggest the normal function of these genes is to suppress central cell proliferation until fertilization, a function that is reflective of the parental conflict theory because both *MEA* and *FIS2* are MEGs (Huh *et al.*, 2008).

More recently, high-throughput genome-wide imprinting studies in *Arabidopsis* resulted in the discovery of additional MEGs and PEGs (Tiwari *et al.*, 2010; Gehring *et al.*, 2011; Hsieh *et al.*, 2011; McKeown *et al.*, 2011; Wolff *et al.*, 2011). These experiments revealed imprinted genes that potentially function to regulate endosperm nutrient flow, embryo growth, and development. Many of these imprinted genes encoded transcription factors, auxin and ethylene signaling related proteins, and components of the ubiquitin-26S proteasome pathway. Epigenetic regulatory proteins important for histone and DNA methylation and small RNA pathway proteins were also found to be imprinted (Hsieh *et al.*, 2011). These findings suggest the parental conflict between maternal nutrient allocation to the embryo manifests at different regulatory levels, including the chromatin level, post-translational level, and at a protein-protein interaction level (Tiwari *et al.*, 2010; Gehring *et al.*, 2011; Hsieh *et al.*, 2011; McKeown *et al.*, 2011; Wolff *et al.*, 2011).

Epigenetic Regulators of *Arabidopsis* Imprinting

DNA Methylation

Eukaryotic DNA methylation is the addition of a methyl group to the 5-carbon of cytosine. The process is enzymatic, carried out by DNA methyltransferases that function to transfer a methyl

group from the universal methyl donor S-adenosyl methionine (SAM) and covalently attach it to the cytosine base, resulting in the formation of 5-methyl cytosine (5mC). This slight modification to the DNA molecule can have a profound effect, sufficient to cause gene silencing. Various models have been proposed to explain the repressive nature of this DNA methylation mark (Cedar and Bergman, 2012). One potential mechanism in which DNA methylation may lead to transcriptional repression is by acting as a blocking agent that physically inhibits the interaction of DNA with transcription factors and other DNA binding proteins essential for transcription (Watt and Molloy, 1988). Alternatively, DNA methylation has also been shown to be strongly associated with the formation of repressive heterochromatin (Klose and Bird, 2006). In this case, the methylation mark is recognized by proteins possessing methyl binding domains (MBDs), which bind to the methylated site and further recruit other proteins, many of which are chromatin remodelers that change the local chromatin architecture into a more heterochromatic state (Cedar and Bergman, 2012).

Eukaryotic DNA methylation is involved in diverse biological processes (He *et al.*, 2011). In mammals, DNA methylation has been shown to be involved in cellular differentiation, X-chromosome inactivation, transposon silencing, promoter silencing, and imprinting (Reik, 2007). In plants, the repressive effects of DNA methylation are used routinely to silence transposable elements, functioning as a genomic defense mechanism (Zemach *et al.*, 2010b). DNA methylation is critical to organism development because null mutations in some of the DNA methyltransferases display embryonic lethality in mammals (Li *et al.*, 1992) and aberrant morphological effects in plants (Zemach *et al.*, 2010b). In both plants and mammals, DNA methylation is critical to genomic imprinting, carrying out allele-specific silencing (Li *et al.*, 1993).

DNA methylation in plants occurs in the contexts of CG, CHG, and CHH (H = A, C, or T) (Cokus *et al.*, 2008). This is strikingly different to the situation in mammals, where DNA methylation occurs overwhelmingly in the CG context, having only a small amount of CHH methylation present in the germline (Ramsahoye *et al.*, 2000; Lister *et al.*, 2009). Studies over the last decade have revealed the varied methylation contexts to be the result of disparate underlying molecular mechanisms, involving distinct DNA methyltransferases, small RNAs (sRNAs), and protein factors (Law and Jacobsen, 2010).

DNA methylation in the CG context is carried out by methyltransferase-1 (MET1), which is a homologue of the mammalian DNA methyltransferase-1 (DNMT1) (Vongs *et al.*, 1993). Similar to its mammalian homologue, MET1 functions to maintain CG methylation in the genome from one mitotic replication to the next. An important component of CG maintenance methylation is the Set or Ring-Associated domain (SRA) containing protein Variation In Methylation 1 (VIM1) in plants, and Ubiquitin-like plant Homeodomain and Ring Finger Domain-1 (UHRF1) in mammals (Law and Jacobsen, 2010). SRA domains are known to bind hemimethylated DNA, and it is believed that UHRF1 recruits DNMT1 to the replication fork as it binds to the nascent hemimethylated CG sites formed after replication (Bostick *et al.*, 2007). The UHRF1 recruitment of DNMT1 to the replication fork restores the two hemimethylated daughter DNA strands to a fully methylated state (Bostick *et al.*, 2007). Null mutations in *Arabidopsis MET1* generate plants with very little CG methylation that display defects in morphology and fertility (Law and Jacobsen, 2010; Zemach *et al.*, 2010b). A key aspect of the establishment of *Arabidopsis* gene imprinting is the MET1-mediated DNA methylation of specific parental alleles during gametogenesis (Huh *et al.*, 2008).

Maintaining CHG methylation in *Arabidopsis* is achieved via the plant-specific DNA methyltransferase CMT3 and its ability to associate with specific histone modifications (Bartee *et al.*, 2001; Lindroth *et al.*, 2001; Johnson *et al.*, 2007). In addition to a methyltransferase domain, CMT3 possesses a chromodomain that binds specifically to histone 3 lysine 9 dimethylation (H3K9Me2) marks (Bartee *et al.*, 2001; Lindroth *et al.*, 2001) suggesting H3K9Me2 may recruit CMT3 for DNA

methylation. The H3K9Me2 mark is established by the histone methyltransferase Supressor of Variegation 3-9 Homologue 4 (SUV4), which contains both a SET and an SRA domain. The SET domain in SUV4 is responsible for dimethylation of H3K9, whereas the SRA domain is believed to bind to methylated DNA. A mechanism for CHG maintenance methylation has been proposed suggesting a reinforcing "loop" between DNA methylation and histone modification, where H3K9me2 marks guide CMT3 to loci to promote DNA methylation, and the DNA methylation marks themselves guide SUV4 to carry out H3K9 dimethylation (Law and Jacobsen, 2010).

The maintenance of CHH methylation is achieved through an elaborate RNAi-mediated process known as RNA-directed DNA methylation (RdDM) (Wassenegger *et al.*, 1994; Henderson and Jacobsen, 2007; Matzke *et al.*, 2009). RdDM is initiated by a plant-specific DNA-dependent RNA polymerase, RNA-Polymerase IV (POLIV), which targets transposons and repetitive elements for transcription, resulting in the production of aberrant single-stranded RNA (ssRNA). The ssRNA serves as a template for RNA replication, carried out by RNA-dependent RNA Polymerase 2 (RDR2) leading to the formation of double-stranded RNA (dsRNA), which is processed into small 24-nucleotide (nt) primary small interfering RNAs (siRNAs) via the Dicer-Like 3 (DCL3) endoribonuclease. The primary siRNAs are further processed by the covalent attachment of a methyl-group onto the 2′-OH group on the 3′-terminal nucleotide of the siRNA, carried out by the SAM-dependent RNA methyltransferase, HUA Enhancer 1 (HEN1). The mature siRNAs are then loaded onto protein complexes that contain Argonaute 4 (AGO4), a member of the Argonaute family of small RNA (sRNA) binding proteins. Argonaute (AGO) proteins are highly conserved across eukaryotes and associate with different classes of sRNAs, such as microRNAs (miRNAs), siRNAs, and the animal related piwi-interacting RNAs (piRNAs) (He *et al.*, 2011). Most AGOs assemble into the RNA-Induced Silencing Complex (RISC), which functions to carry out post-transcriptional gene silencing (Czech and Hannon, 2011). However, in the case of RdDM, the siRNA-loaded AGO4 (siRNA/AGO) further recruits an additional component, RNA Polymerase V (POLV). POLV, similar to POLIV, is a plant-specific DNA-dependent RNA polymerase; however, the role of POLV in RdDM is to transcribe noncoding RNA transcripts from intergenic regions. The function of the POLV-transcript is to tether the siRNA/AGO complex near the DNA, enabled by base-pair complementarity between the AGO-associated siRNA and POLV transcript. The RNA-binding protein Kow Domain-Containing Transcription Factor 1 (KTF1) is then recruited to the nascent complex, further strengthening this base-pair complementarity (Schwartz and Pirrotta, 2007) and finalizing the formation of the mature RdDM-effector complex. The RdDM-effector complex stimulates the recruitment of the *de novo* methyltransferase Domains Rearranged Methyltransferase 2 (DRM2), which establishes and maintains DNA maintenance methylation in the CHH context (Law and Jacobsen, 2010).

Histone Modifications

The histone tails emanating from the core nucleosomes of chromatin are subject to numerous reversible chemical modifications, including methylation, demethylation, acetylation, deacetylation, phosphorylation, ubiquitinylation, sumolyation, and ADP-ribosylation (Kouzarides, 2007). These specific modifications cause structural changes to chromatin architecture and to the genes residing within, leading to transcriptional activation or repression depending on the type of modification (Kouzarides, 2007).

The histone modification most critical to plant imprinting is the trimethylation of H3K27, which leads to transcriptional repression (Pirrotta, 1998; Schwartz and Pirrotta, 2007, 2008). H3K27me3

is carried out by the Polycomb group complex (PcG), a 600-kDa multiprotein complex originally discovered as a major regulator of homeotic *Hox* genes in *Drosophila*. *Hox* genes are responsible for setting up the fundamental body plan and segmentation of the developing fruit fly, and the repressive function of the PcG complex ensures their correct temporospatial expression. Null mutations in members of the PcG protein complex cause striking developmental abnormalities in *Drosophila*, such as the ectopic placement of sex combs on the second and third legs of the adult male fly (Grimaud *et al.*, 2006). *Drosophila* PcG proteins are found in two distinct complexes: PcG Repressive Complex 1 (PRC1) and PcG Repressive Complex 2 (PRC2). However, only the PRC2 has been found in *Arabidopsis* (Gehring *et al.*, 2006; Huh *et al.*, 2008).

In *Arabidopsis*, there are several PRC2 variants that are key players in numerous developmentally related processes, such as the change from vegetative phase to reproductive phase, vernalization, flowering time, endosperm development, and imprinting (Hennig and Derkacheva, 2009). The *Arabidopsis* PRC2 involved in endosperm imprinting (also called the FIE-MEA complex) consists of the SET-domain containing protein Medea (MEA), a C2H2 zinc-finger protein Fertilization Independent Seed 2 (FIS2), and two WD-40 proteins Fertilization Independent Endosperm (FIE) and Multicopy Supressor of IRA 1 (MSI1) (Hennig and Derkacheva, 2009). Similar to its *Drosophila* homologue, PRC2 in *Arabidopsis* appears indispensable for development because null mutations in the PRC2 component genes *MEA, FIE, FIS2*, and *MSI1* all cause dramatic developmental abnormalities (Huh *et al.*, 2008; Hennig and Derkacheva, 2009). As described subsequently, PRC2 in *Arabidopsis* acts to establish the imprinting of many genes.

Active DNA Demethylation

DNA demethylation occurs in many organisms both as a passive and as an active process. Passive DNA demethylation is the removal of 5mC by a failure of the DNA methylation maintenance machinery to methylate a particular cytosine during replication. Continual replication in the absence of DNA methylation gradually dilutes the methylation mark, until eventually methylation is absent. The process of active DNA demethylation involves the enzymatic removal of 5mC (He *et al.*, 2011). Because DNA methylation functions as a repressive epigenetic mark, one could surmise that demethylation would lead to transcriptional derepression (activation). Active DNA demethylation is functionally antagonistic to DNA methylation, generally resulting in gene expression (Ooi and Bestor, 2008). Over the last decade, a considerable amount of work has gone into elucidating the mechanisms of active DNA demethylation.

DNA Glycosylases and Base Excision Repair Pathway

DNA glycosylases function to remove damaged or mispaired bases from genomic DNA and are the first enzymes in the the base excision repair (BER) pathway (Jacobs and Schar, 2012). A damaged base is initially recognized by a DNA glycosylase, which flips the base out from the double helix and hydrolyzes the N-glycosidic bond (ribose-base), resulting in removal of the base from the double helix. This produces an apurinic/apyrimidinic (AP) site, which is further processed by an AP lyase that nicks the sugar-phosphate backbone. The single-strand break in the sugar-phosphate backbone activates an AP endonuclease to create a 3′-hydroxyl, allowing a DNA polymerase to add the correct base. A DNA ligase is then used to seal the nick, completing the BER pathway.

Active DNA Demethylation Is Carried out by 5mC-Specific DNA Glycosylases and Components of BER Pathway

In *Arabidopsis*, active DNA demethylation is carried out by the DEMETER (DME) family of DNA glycosylases and components of the base excision repair (BER) pathway (He *et al.*, 2011). In

contrast to other DNA glycosylases, the primary function of the DME glycosylases is to remove 5mC, instead of damaged or mispaired bases (Choi *et al.*, 2002; Gehring *et al.*, 2006). The methylated cytosine is flipped out of the double helix, and its N-glycosidic bond is hydrolyzed by the DME DNA glycosylase enzyme, resulting in the expected AP site and a free methylated base (5mC). DME further nicks the sugar-phosphate backbone at the AP site, as a consequence of being a bifunctional helix-hairpin-helix DNA glycosylase, which has both DNA glycosylase and AP lyase activity (Gehring *et al.*, 2006). At this point, downstream components of the BER pathway are activated (i.e., AP endonuclease, DNA polymerase, and DNA ligase), resulting in replacement of 5mC with unmethylated cytosine.

The DME family of DNA glycosylases consists of Demeter (DME), Repressor Of Silencing 1 (ROS1), Demeter-Like 2 (DML2), and Demeter-Like 3 (DML3). Each has different functions in *Arabidopsis*, and *ROS1*, *DML2*, and *DML3* are expressed broadly throughout the plant, mainly in adult vegetative tissue. A genome-wide methylation analysis of whole plant tissue possessing a triple mutation in *ros1 dme2 dml3* found several hundred hypermethylated loci compared with wild-type tissue, suggesting they are targeted by these three DNA glycosylases in wild-type plants (Penterman *et al.*, 2007; Lister *et al.*, 2009). Most of these demethylated loci appeared at the 5′ and 3′ ends of genes, a pattern opposite to that of DNA methylation. A model was proposed suggesting the three DNA glycosylases worked to "prune" away DNA methylation from the ends of genes. In essence, this 5′ and 3′ gene-adjacent demethylation is proposed to act to remove misdirected DNA methylation that may have made its way into transcriptionally sensitive areas of genes (Penterman *et al.*, 2007; Zhu *et al.*, 2007).

DME Is a Reproductive Companion Cell Demethylase Critical to Imprinting

In contrast to ROS1, DML2, and DML3, which are broadly expressed throughout the plant, DME has been shown to be primarily expressed in reproductive tissues, specifically the companion cells of the developing female and male gametophytes (Choi *et al.*, 2002; Schoft *et al.*, 2011). DME-mediated DNA demethylation occurs in the central cell of the female gametophyte before fertilization, resulting in the maternal expression of several genes (Gehring *et al.*, 2006; Huh *et al.*, 2008). Activity of DME in the central cell is essential for setting up the allele-specific methylation patterns that ultimately establish genomic imprinting in the *Arabidopsis* endosperm (Choi *et al.*, 2002; Gehring *et al.*, 2006). Central cell DME-mediated DNA demethylation is required for subsequent seed development because seeds inheriting a maternal *dme*-mutant allele are nonviable. Thus, *dme*-mutant heterozygous plants generate seeds in which half of the progeny abort, and the other half are normal (Choi *et al.*, 2002).

DME is also expressed in the vegetative cell of the male gametophyte (Schoft *et al.*, 2011). Similar to the *dme*-mutant female gametophyte, a male pollen inheriting a *dme*-mutant allele also displays a mutant phenotype in the form of an inability to germinate properly. Locus-specific methylation analysis revealed that the vegetative cell genome has several loci targeted by DME for DNA demethylation. These loci were the same as the loci in the central cell, suggesting DME may be targeting similar loci in both the central cell and the vegetative cell for active DNA demethylation (Schoft *et al.*, 2011).

Mechanisms Establishing *Arabidopsis* Gene Imprinting

Until recently, there were only ten known imprinted genes in *Arabidopsis* (Bauer and Fischer, 2011). Many genes have been studied in great detail, leading to the elucidation of three key

epigenetic strategies that are used to promote parent-of-origin monoallelic silencing: (1) DME-mediated DNA demethylation, (2) MET1-mediated DNA methylation maintenance, and (3) PRC2-mediated transcriptional repression (Bauer and Fischer, 2011). The recent expansion in the number of known *Arabidopsis* imprinted genes provides many more genes whose imprinted expression regulation may be studied (Tiwari *et al.*, 2010; Gehring *et al.*, 2011; Hsieh *et al.*, 2011; McKeown *et al.*, 2011; Wolff *et al.*, 2011). Data from a genome-wide imprinting study show many of these genes fall into one of the three established regulatory groups; however, numerous genes use apparently novel silencing mechanisms (Hsieh *et al.*, 2011).

MEGs Established by DME-Mediated DNA Demethylation

FWA is maternally expressed and paternally silenced in the endosperm, and its imprinted status in *Arabidopsis* has been known for some time. Using expression of a *pFWA::FWA-GFP* transgene, it was shown that *FWA* is expressed only in the central cell and endosperm (Kinoshita *et al.*, 2004). The ectopic expression of FWA protein, a homeodomain-containing transcription factor, causes a late flowering phenotype (Soppe *et al.*, 2000). In the endosperm, an upstream region of the promoter for the maternal *FWA* allele is hypomethylated compared with the paternal *FWA* allele and maternal and paternal *FWA* alleles in other tissues (embryo, seed coat, leaf, and pollen). The hypomethylated maternal *FWA* allele is expressed whereas the hypermethylated paternal *FWA* allele is not, resulting in monoallelic expression in the endosperm. As expected, a *MET1* mutation in the pollen donor results in biallelic *FWA* expression in the endosperm, whereas mutations in *DME* in the female gametophyte result in silencing of the maternal *pFWA::GFP* allele (Kinoshita *et al.*, 2004). Another imprinted gene in *Arabidopsis*, Polycomb-group protein *FIS2*, is regulated in a similar way. An imprinting regulation model can be made from such data; *MET1* maintains 5′ CG methylation in the promoter region of the paternal allele, whereas *DME*, a gene that is expressed in the central cell and not in sperm, demethylates and activates the maternal allele (Figure 4.1*A*). DNA methylation–mediated repression of the paternal allele results, whereas DME-mediated demethylation of the maternal allele results in expression of the maternal allele after fertilization (Jullien *et al.*, 2006).

One can predict that other maternally expressed genes whose regulation is consistent with the above-described model will display biallelic expression in a paternal *met1* background, whereas its expression would be downregulated in a maternal *dme* background. Genome-wide allele-specific transcriptome studies identified nine new imprinted genes that are in accord with these predictions (Table 4.1). Although the number of new imprinted genes is small, they potentially play an important role in endosperm development because their functions indicate they may act to regulate the expression of other genes (Hsieh *et al.*, 2011).

MEGs Established by DME-Mediated DNA Demethylation and PRC2 Repression

The maternally expressed gene *MEA* uses a more complex imprinting expression mechanism. DME in the central cell demethylates CG sites flanking *MEA*, which is required for *MEA* maternal expression. Because paternal *met1* did not release silencing of the *MEA* paternal allele, it is known that *MEA* regulation is not simply dependent on DNA methylation. Instead, mutation of the maternal *FIE* allele (or other components of PRC2) resulted in biallelic expression of *MEA* in the endosperm (Gehring *et al.*, 2006). Another maternally expressed gene, *AfH5*, exhibits similar behavior; its promoter is a target for PRC2, and mutation of maternal *MEA* releases *AfH5* paternal silencing

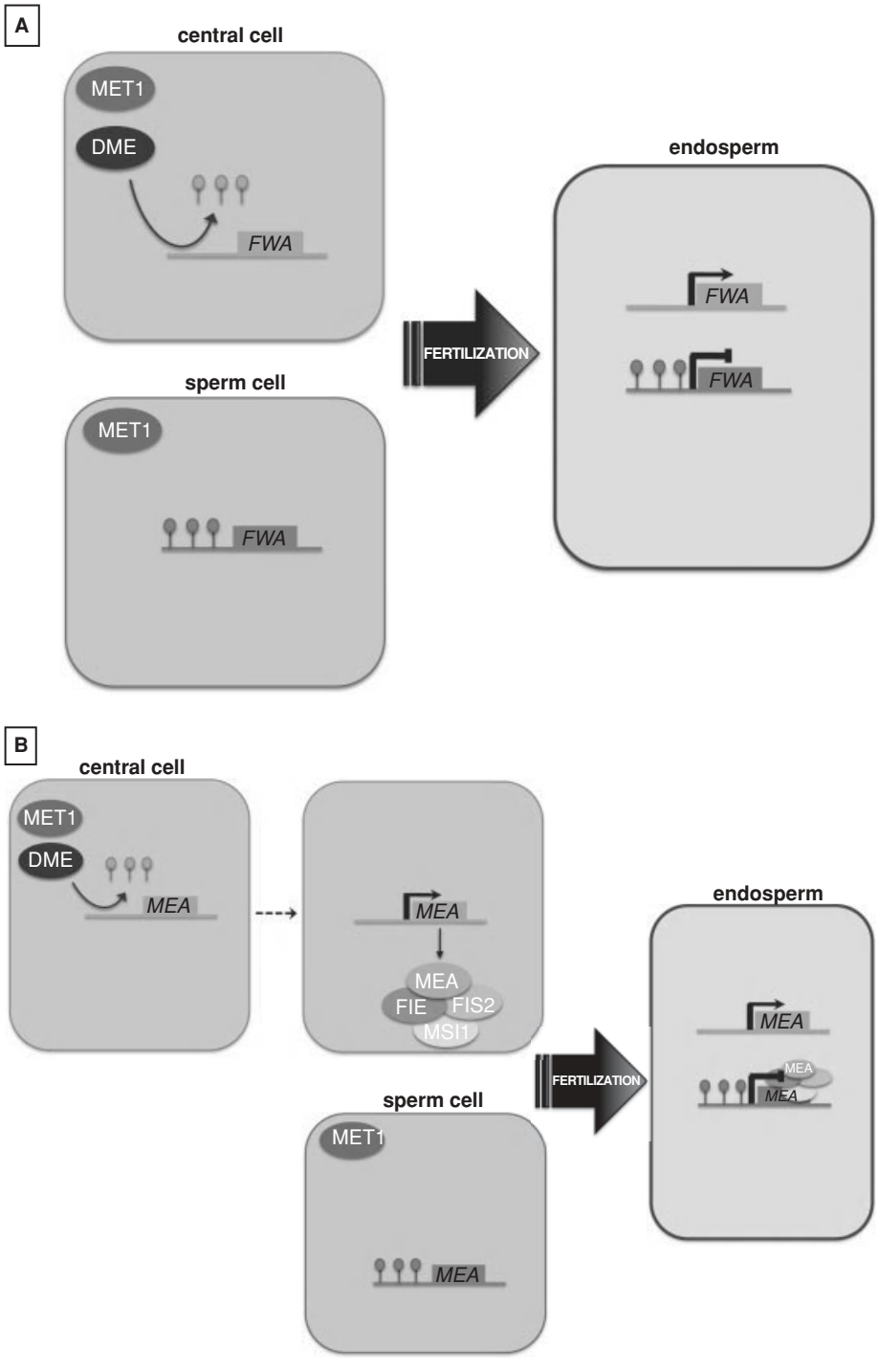

Figure 4.1 *A–C,* Models for *Arabidopsis* genomic imprinting: *FWA* model *(A)*, *MEA* model *(B)*, and *PHE1*-model *(C)*. "Lollipops" represent 5-methyl cytosines. The red bar on the maternal *PHE1* allele in represents the location of the 3′-tandem repeats targeted for DME-mediated DNA demethylation. (For color detail, see color plate section.)

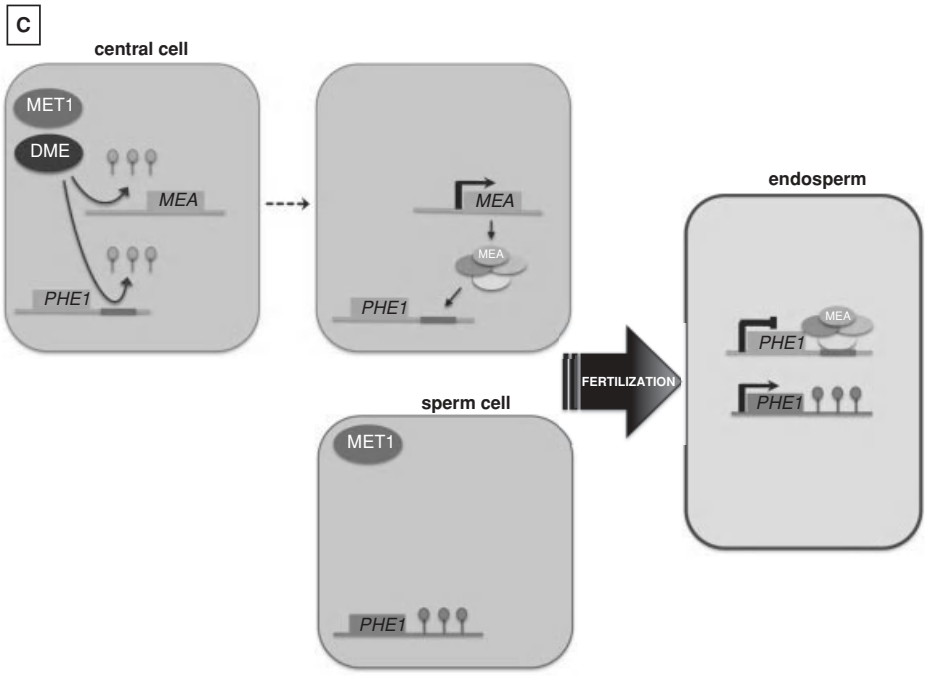

Figure 4.1 (*Continued*)

(Gerald *et al.*, 2009). The model proposed to account for the regulation of these two genes is that before fertilization, DME demethylates and activates *MEA* in the central cell. The *MEA* protein then forms part of the PRC2 complex with *FIE*. After fertilization, in the endosperm, the PRC2 complex silences the paternal allele of imprinted genes, presumably through enrichment of repressive histone modifications on this allele (Figure 4.1*B*).

For imprinted genes that follow the DME/PRC2 model, it is expected that paternal allele silencing will not be disrupted by paternal *MET1* mutations but will be affected by mutations in maternal *FIE*. In addition to *MEA* and *AfH5,* 20 new genes have been found more recently to follow this expression pattern (Table 4.2). Most of these genes were found to have functions related to intermediary metabolism or signaling. However, only two of these genes, *At1g69900* and *At5g47770*, became biallelic in the maternal *DME* mutant, as was the case for *MEA*. Because DME is needed for *MEA* and *FIS2* (maternal) expression, this means that 18 of the new FIE-related MEGs are paternally

Table 4.1 New MEGs Established by DME-Mediated DNA Demethylation

ATMYB3R2	at4g00540	Transcription factor
ERF/AP2	At4g31060	Transcription factor
–	At1g59930	PHERES-like imprinted transcription factor-related (truncated)
JLO	At4g00220	Transcription factor, regulates PIN expression for auxin transport
EIN2	At5g03280	ethylene hormone signaling
DRB2	At2g28380	DS RNA-binding, Component of sRNA pathway
SUVH8	At2g24740	SET-domain protein (H3K9 methyltransferase)
SDC	At2g17690	F-box (targets protein for ubiquitylation, sending it to the 26S proteasome)
MRU1	At5g35490	Unknown function. Overexpressed in non-CG met mutant

Table 4.2 New MEGs Regulated by DME-Mediated DNA Demethylation and PRC2 Complex

–	At1g08050	Zinc finger family (C3HC4-type RING finger)
–	At1g21790	TRAM, LAG1 and CLN8 lipid-sensing domain containing protein
–	At1g69900	Actin cross-linking protein
–	At1g76250	–
–	At1g76820	eukaryotic translation initiation factor 2 family binding to GTP, have GTPase activity
AtSKP2	At1g77000	Homolog to human SKP2, may be involved in degradation of a CDK inhibitor
–	At2g17990	–
ADS2	At2g31360	Homolog to acyl desaturase in cyanobacteria, yeast and mammals.
–	At3g17250	PP2 (Protein phosphastase 2C)-related
–	At3g22810	PI (Phosphoinositide-binding) protein involved in signal transduction
AtTKP5	At4g01840	K^+ channel family protein
ACX1	At4g16760	Acyl-CoA oxidase involved in jasmonate biosynthesis
CAND6	At5g02630	Lung seven transmembrane receptor family
–	At5g03370	Acylphosphatase
–	At5g22920	(CHY-type) Zinc finger protein
–	At5g24460	–
–	At5g42235	Defensin-like (DEFL) family protein
FPS1	At5g47770	Farnesyl diphosphate synthase1 involved in isopropenoid biosynthesis
AGP22	At5g53250	Arabinogalactan protein22
ENODL1	At5g53870	Early nodulin-like protein1 that carry electron in plasma membrane

silenced in the absence of MEA and FIS2 components of PRC2. One possible explanation for the more limited effect of the *dme* mutation compared with the *fie* mutation is that *FIE* is a single-copy gene required for all PRC2 complex formation (Ohad *et al.*, 1999), whereas MEA and FIS2 are both interchangeable with other PcG family members (i.e., Swinger and Curly Leaf for MEA; Vernalization 2, and Embryonic Flower 2 for FIS2), which might provide redundant PRC2 functionality in a *dme* mutant background.

PEGs Established by DME-Mediated DNA Demethylation and PRC2 Repression

PHERES1 (PHE1) was the first paternally expressed imprinted gene to be discovered in *Arabidopsis*, and its analysis resulted in a paradigm for paternal-specific expression (Kohler *et al.*, 2012). *PHE1* is a type I MADS-box transcription factor that is expressed after fertilization in the chalazal endosperm and repressed in other parts of the seed by PRC2. Mutation of *PHE1* does not result in seed abortion, but *phe1* partially rescues *mea*, suggesting that the seed abortion phenotype of *mea* is partially due to the failure of PRC2 to reduce the dosage of *PHE1* through transcription of only one allele (Kohler *et al.*, 2012). Disruption of the DNA methylation pattern on the maternal *PHE1* allele did not reactivate the silenced maternal allele. On the contrary, paternal *MET1* mutations decreased paternal *PHE1* expression after fertilization. The silent maternal *PHE1* allele is maintained by PRC2, and loss of maternal PRC2 results in loss of *PHE1* imprinting (Kohler *et al.*, 2005). DNA methylation of tandem repeats located 2 kb downstream (3′ side) of the *PHE1* coding region is required for *PHE1* expression (Villar *et al.*, 2009). A model was proposed suggesting the downstream *PHE1* repeats on the maternal allele are demethylated by DME in the central cell, allowing the binding of PRC2 and repression of the *PHE1* maternal allele. In contrast, the paternal allele is expressed because its 3′-tandem repeats remained methylated, preventing PRC2 binding (Figure 4.1*C*) (Makarevich *et al.*, 2006).

Table 4.3 New PEGs Regulated by DME-Mediated DNA Demethylation and PRC2 Complex

–	At2g36560	Contain DNA binding domain
YUC10	At1g48910	Flavin monooxygenase related to auxin biosynthesis
VIM5	At1g57800	maintenance of DNA methylation
–	At4g11940	–
–	At5g63740	–
SUVH7	At1g1770	SET-domain protein (H3K9 methyltransferase)

PEGs that follow the *PHE1* model would be expected to become biallelically expressed in endosperm inheriting a maternal *fie* and *dme* and downregulated in endosperm inheriting a paternal *met1* mutant allele. Among nine recently identified PEGs, all but one (VIM5) became biallelically expressed in *fie* endosperm and downregulated in *met1* (Table 4.3). Five genes showed biallelic expression in *dme,* whereas three genes, *At1g31640 (AGL92)*, *At1g660410* (encodes an F-box protein), and *At2g21930* (encodes an F-box protein), are still imprinted in *dme* mutant endosperm. This finding suggests that most PEGs are regulated in a manner similar to *PHE1*. *VIM5*, related to *VIM1* and expressed only in the endosperm, seems to be strictly regulated by PRC2, whereas *At1g660410* and *At2g21930* are not regulated by *DME*.

Imprinting in the Embryo

Although genes with imprinted expression in the endosperm are plentiful, there are only a few examples of imprinted genes in the plant embryo. A study by Jahnke and Scholten (2009) showed that in reciprocal crosses of different maize inbred lines, only the maternal transcripts of *maternally expressed in embryo 1 (mee1)* are detected in both embryo and endosperm. The expressed maternal alleles of *mee1* correlate with hypomethylation in the promoter and coding sequences of the *mee1* gene. The silent paternal alleles are hypermethylated at both CG and CHG contexts suggesting that allele-specific differential methylation plays a role in the imprinted expression of *mee1* in the embryo and endosperm. The *mee1* gene is demethylated and expressed in the maize central cell but methylated and silent in both the sperm and the egg cell. On fertilization, the maternal *mee1* allele rapidly becomes demethylated in the embryo, whereas the paternal allele remains methylated. This observation suggests the presence of epigenetic marks other than DNA methylation that allow the zygote to differentiate between the parental alleles of this gene, selecting only the maternal *mee1* allele for active demethylation. In contrast to the situation in the endosperm, differential methylation of the two parental *mee1* alleles in the embryo does not persist throughout embryogenesis, and by 16 days after pollination, the maternal *mee1* allele has become methylated and silent.

The occurrence of imprinted expression during early embryo development, although rare, might not be specific to maize. Nodine and Bartel (2012) used high-throughput RNA-sequencing techniques to sequence the transcripts of hybrid embryos from crosses between two polymorphic *Arabidopsis* accessions. They found that despite equal contribution from both parental genomes for 1- to 32-cell stage embryos, a small group of genes clearly display parentally biased expression patterns (77 maternally enriched genes and 45 paternally enriched genes) (Nodine and Bartel, 2012). It is thought that the imprinted expression of these genes in early *Arabidopsis* embryogenesis does not persist in later seed development because similar genome-wide high-throughput sequencing approaches assessing imprinted genes at later stages of seed development identified only very few parentally biased transcripts in the embryo (Gehring *et al.*, 2011; Hsieh *et al.*, 2011).

Imprinting in Monocots

More recent high-throughput imprinting studies in *Zea mays* (Waters *et al.*, 2011; Zhang *et al.*, 2011) and *Oryza sativa* (Luo *et al.*, 2011) have greatly increased the number of imprinted genes in these agriculturally important monocots. Similar to *Arabidopsis*, gene imprinting in cereals almost exclusively occurs in the endosperm (Jahnke and Scholten, 2009).

Only a few genes were found to be mutually imprinted in *Arabidopsis*, maize, and rice (Waters *et al.*, 2011). However, because only one third to one half of the genes assessed in each species were analyzed for imprinting (a consequence of SNP availability and read coverage (Waters *et al.*, 2011)), conclusions made from cross-species comparisons derived from gene homology are limited. Nonetheless, one difference between the seeds of monocots and dicots is the development of their endosperm: monocots have a persistent endosperm, which remains surrounding the embryo until germination, whereas dicots have a nonpersistent endosperm that is almost completely gone by the time of germination (Olsen, 2004). Potential differences in the types of imprinted genes between *Arabidopsis*, rice, and maize may be a reflection of the developmental pathway of their respective endosperm.

An association between DNA methylation and imprinting has been observed in both rice and maize (Springer and Gutierrez-Marcos, 2009). Additionally, in maize, strong correlations have been made between the epigenetic marks of DNA methylation and H3K27me3 and gene imprinting (Springer and Gutierrez-Marcos, 2009; Raissig *et al.*, 2011). Finally, maize may also use active chromatin modification in the form of H3 and H4 acetylation (H3/H4Ac) to regulate imprinting (Haun and Springer, 2008). Active DNA demethylation has yet to be genetically identified in monocots, although *in silico* investigation has identified orthologs of the DME family of DNA glycosylases in rice (Zemach *et al.*, 2010a) and likely maize. Rice plants with a null mutation in a rice DME-ortholog, *ROS1a*, display reproductive phenotypes similar to phenotypes observed in *Arabidopsis dme* mutant plants (Ono *et al.*, 2012). Taken together, these findings suggest the mechanisms used to establish plant gene imprinting may be common between monocots and dicots.

Imprinting in Rice

Previous studies in rice endosperm led to the discovery of two imprinted genes, *OsFIE1* (Luo *et al.*, 2009) and *OsMADs87* (Ishikawa *et al.*, 2011). *OsFIE1* is a MEG specifically expressed in the endosperm that is related to the imprinted maize *FIE1* gene (Luo *et al.*, 2009). In *Arabidopsis*, null mutations in *FIE* result in an overproliferated endosperm. However, a T-DNA insertion in *OsFIE1* showed no such phenotype, suggesting imprinted *OsFIE1* in rice is not involved in the repression of central cell development (Luo *et al.*, 2009). The *OsMADs87* gene in rice was found to be a homologue of the paternally expressed *Arabidopsis* imprinted gene, *PHE1*. However, in rice, *OsMADs87* is a maternally expressed imprinted gene (Ishikawa *et al.*, 2011), as opposed to the paternal expression of *PHE1* in *Arabidopsis*. The function of *OsMADs87* in the endosperm is unknown.

A high-throughput transcriptome study conducted in rice brought the current number of known imprinted genes to 121, of which 62 are MEGs and 59 are PEGs (Luo *et al.*, 2011). All gene imprinting was found in the endosperm except for one gene, *Os10g05750*, which was a MEG in both the endosperm and the embryo. *Os10g05750* encodes an allergenic protein in olive (*Olea europaea* L.) that is thought to control pollen tube emergence and guidance (de Dios Alche *et al.*, 2004). Ontological analysis of imprinted rice genes show 30% of them to be of unknown function,

whereas the remaining genes were suggested to be involved in regulatory processes such as signal transduction, cellular component organization, and DNA and RNA binding (Luo *et al.*, 2011).

The imprinted rice dataset was further compared with some more recent *Arabidopsis* high-throughput imprinting studies (Gehring *et al.*, 2009, 2011; Hsieh *et al.*, 2011; Wolff *et al.*, 2011) to find genes with similar homology. Overall, when using the filtered data presented in the literature, a low-degree of conservation was detected between the rice and *Arabidopsis* imprinted gene datasets. A low level of conservation could suggest that plant gene imprinting evolved rapidly and independently in monocots and dicots. Of the 27 candidate genes that showed significant homology between the rice and *Arabidopsis* datasets, several have key functions in the sRNA pathway (*AGO* and *DsRNA BINDING*), chromatin remodeling (*SUVH* and *PICKLE-LIKE*), and DNA methylation (*VIM5*) (Luo *et al.*, 2011). If imprinting did evolve independently, the dosage attenuation of these common, key regulatory genes must be an important process. Alternatively, as previously mentioned, the lack of conservation between the imprinted gene datasets of rice and *Arabidopsis* may be due to the technical limitation of available SNPs or inappropriate data filtering.

Imprinting in Maize

Before more recent high-throughput approaches, earlier studies in *Zea mays* resulted in identification of a few imprinted genes. *fie1* and *fie2* are MEGs found in the endosperm and are homologues of *Arabidopsis FIE* (Springer *et al.*, 2002; Danilevskaya *et al.*, 2003; Gutierrez-Marcos *et al.*, 2003). *fie1* is expressed solely in the endosperm, displaying "binary" imprinting throughout seed development. Binary imprinting is defined to be when one parental allele is completely silenced, contrasting with "differential" imprinting when only a relative difference between parental allele expression is observed (Gutierrez-Marcos and Springer, 2009). In the case of *fie2*, expression is more ubiquitous, occurring in both reproductive and vegetative tissues, and is imprinted only during the early stages of endosperm development (Danilevskaya *et al.*, 2003; Gutierrez-Marcos *et al.*, 2003, 2006). Null-mutations in *fie1* and *fie2* have not yet been identified, and consequently the function of these two genes has not been determined.

meg1 is a MEG that displays imprinting during the early stages of endosperm development (Gutierrez-Marcos *et al.*, 2004). Notably, *meg1* imprinting occurs in the basal transfer region of the endosperm, in the transfer cells that function to facilitate the movement of nutrients from the maternal tissues of the sporophyte to the endosperm (Olsen, 2004). *meg1* encodes a novel protein that was shown to be glycosylated and localized to the cell wall ingrowths of the basal transfer cells (Gutierrez-Marcos *et al.*, 2004). A more recent study by Costa *et al.* (2012) has further demonstrated *meg1* to be required for transfer cell formation and differentiation. Maternally expressed imprinted *meg1* was also shown to be essential in regulating maternal nutrient uptake, sucrose partitioning, and increasing seed biomass yield, as a *meg1*-null mutant produced smaller seeds (Costa *et al.*, 2012). These results indicate that maternally expressed *meg1* promotes nutrient allocation to the embryo instead of suppressing it – a finding that is not in accord with the parental conflict theory (Haig, 2000). Costa *et al.* (2012) suggested imprinting may not have arisen from parental conflicts over nutrient amounts to offspring (Moore and Haig, 1991) but instead from maternal-offspring influences over placental-like functions. In this case, the evolutionary drive causing imprinting seems more conducive to a coadaptation theory of genomic imprinting (Wolf and Hager, 2006).

The *nrp1* gene is another MEG in maize, exclusively expressed in the endosperm (Guo *et al.*, 2003). The function of *nrp1* is unknown, but it encodes a putative transcription factor of the NAM gene family.

A genome-wide transcriptome study conducted by Waters *et al.* (2011) has increased the number of known potentially imprinted genes in maize to ~100, of which 54 are MEGs and 46 are PEGs. Gene ontology analysis of the imprinted genes showed an enrichment for orthologs of chromatin-related proteins and transcription factors, such as *VIM1* (Woo *et al.*, 2008), *VERNALIZATION 5* (Greb *et al.*, 2007), *FIE* (Ohad *et al.*, 1999), and *CURLY LEAF* (Goodrich *et al.*, 1997).

Evolution of Plant Imprinting

Similarities between mutant phenotypes, imprinting mechanisms, and sites of gene imprinting (placenta and endosperm) support the idea of convergent evolution of genomic imprinting in plants and mammals (Feil and Berger, 2007) and provide persuasive evidence that the seminal theory of Moore and Haig (1991) may be correct. The facts that angiosperms contain an organ synonymous to the placenta and that they also imprint genes are striking. That the blunt method of altering genomic ploidy results in a change in seed size is consistent with the parental conflict theory in *Arabidopsis* and maize. The generation of maize endosperm that departs from the normal 2:1 ratio between female and male genomes exerts a dramatic effect on seed development. Paternal genomic excess prolongs early endosperm development and delays cellularization, resulting in larger seeds, whereas maternal genome excess results in the opposite phenotype, accelerating the onset of cellularization and resulting in smaller seeds (Lin, 1984; Lin and Dickinson, 2010). Likewise, in *Arabidopsis*, doubling the maternal genome contribution results in a smaller endosperm and embryo, and doubling the paternal contribution results in a larger endosperm and embryo (Scott *et al.*, 1998). These results support the idea that gene imprinting in monocots and dicots is intimately involved with resource allocation to the embryo.

Seed size itself is not a barrier to maternal fitness in the same way as it is to placental mammals. More likely, the nutrient transfer capacity of the endosperm is key in the balance of parental resources. In maize, the previously mentioned maternally expressed imprinted *meg1* gene was shown to be fundamental for the development of the endosperm nutrient transfer cells that are at the interface between the mother and the seed (Gutierrez-Marcos *et al.*, 2004). *meg1* was found to regulate maternal nutrient uptake, sucrose partitioning, and seed biomass; however, its maternal expression is at odds with the predictions of parental conflict.

An alternative, although not mutually exclusive, hypothesis is that plant gene imprinting is a by-product of transposable element (TE) silencing. Most imprinted genes in *Arabidopsis* and rice are flanked by transposable elements (TEs). DNA demethylation appears to occur at TEs that flank *Arabidopsis* imprinted genes (Gehring *et al.*, 2009; Hsieh *et al.*, 2009; Schoft *et al.*, 2011). It is possible that there is an underlying requirement for DNA demethylation in companion cells (central cell and vegetative cell) to ensure the RdDM-mediated methylation of TEs in the gametes (Schoft *et al.*, 2011). The fact that DNA demethylation of central and vegetative cells may be similar, despite their dramatically different functions in plant reproduction, argues that TE silencing, rather than gene imprinting, may be the basal function of DNA demethylation in plant reproduction.

Kohler *et al.* (2012) examined unfiltered next generation sequencing data for imprinted genes and found that a higher level of conservation potentially exists in both the genes and the function of genes imprinted in rice, maize, and *Arabidopsis*. These clades are separated by ~150 million years, similar to the divergence of humans and marsupials. Therefore, it remains plausible that imprinting has a specific purpose (Kohler *et al.*, 2012). Although DNA demethylation may have initially evolved to ensure TE silencing, its persistence may also be due to the benefit conferred through dosage attenuation of particular genes involved in seed development and nutrient allocation.

Conclusion

Plant genomic imprinting has been demonstrated in both dicots and monocots, occurring almost exclusively in the endosperm. The parental conflict theory suggests that gene imprinting arose to mediate the competition between parents regarding the level of maternal nutrients and resources allocated to the offspring. This conflict is predicted to occur in the tissues that deliver nutrients to the offspring. However, the findings of maternally expressed *meg1* in maize functioning to promote resource allocation to the embryo suggests a maternal-offspring coadaptation model for imprinting may be a driving force for imprinting as well. Whether or not these different selective forces for imprinting overlap or are mutually exclusive remains to be determined. Nevertheless, gene imprinting in both mammals and plants occurs in structures at the interface between the maternal circulation and the embryo – in mammals, it is the placenta, whereas in plants, it is the endosperm, and both organs act to regulate nutrient exchange.

Epigenetic mechanisms in the form of DNA methylation and histone modifications are the molecular factors responsible for carrying out imprinting in both mammals and plants. However, plants have an additional mechanism in the form of active DNA-demethylation, which is performed by the DME family of plant-specific DNA glycosylases. DME-mediated DNA demethylation involves a co-opting of the BER pathway that results in the removal of 5mC. Notably, DME-mediated DNA demethylation effectively causes the transcription of genes and appears to be an integral part of imprinting regulatory strategies for genes.

High-throughput next generation sequencing has advanced the field of plant gene imprinting, leading to the discovery of more imprinted genes not only in the model plant *Arabidopsis* but also in economically relevant crop plants, rice and maize. The discovery of new plant imprinted genes reveals these genes to be from a range of ontological classes, and the function of dosage attenuation in these proteins provides exciting new avenues of research. Current data suggest that monocot species use DNA methylation and histone modifications to establish genomic imprinting in the same way as *Arabidopsis*, indicating that imprinting and its regulatory strategies are conserved in these highly diverged organisms. Further work must be done to investigate imprinting regulation in these species.

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5 Apomixis

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Introduction

Food security is a challenge for the future of our planet with global population expected to reach 9 billion by 2050. Food production must increase on diminishing arable land, regardless of changing climate pressures. Societal expectations of modern agribusiness also demand decreased dependence on agrichemicals and increased stewardship of land and water resources, conserving them for future generations (Tester and Langridge, 2010).

Hybrid crops exhibit superior yields (15%–35%), attributed to the little understood phenomenon of heterosis or hybrid vigor (Kempe and Gils, 2011). In addition to increased yield, hybrids display improved physical stability, higher responses to fertilizers, better root penetration, and improved tolerance to drought and heat (Kempe and Gils, 2011). However, the production and use of hybrids in world food production are restricted to a few crops, such as maize, sorghum, sunflower, cucumber, onion, and some varieties of rice. Hybrid yield advantages, increased genetic diversity, and accompanying agronomic trait gains from other significant crops including wheat and soybean, currently generated from homozygous inbred varieties, are yet to be realized. The multiple benefits of hybrids come at the cost of expensive and often technically demanding commercial hybrid seed production methodologies. For example, the cross may need to be carried out in a particular direction, and means to prevent seeds forming from the parents can be technically difficult and labor intensive. Parental lines must be maintained so that hybrid seed can be continuously remade, because seeds from high yielding hybrids cannot simply be resown to generate improved crops in subsequent generations; this is due, in part, to the intrinsic nature of sexual reproduction that drives genetic diversity. The combination of meiosis during male and female gamete formation and subsequent gamete fusion at fertilization to generate the embryo lead to segregation of alleles in the progeny, and a coincident decline in heterosis is observed in subsequent seed generations. Economic hybrid seed production coupled with heterotic stability and, preferably, preservation of the hybrid seed genotype would accelerate wider use of hybrids in agriculture.

Some plants naturally produce seeds asexually by a suite of mechanisms under the collective term of apomixis. Apomixis influences reproductive development within the ovule; male gamete development is generally not affected. In contrast to sexual seed formation, apomictic reproduction avoids meiosis and fertilization to generate an embryo that is solely maternal in genotype. The maternal genotype remains fixed and is stably maintained, barring mutation, from one seed generation to the next. Application of apomixis in hybrid seed production would enable hybrid genotype fixation

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and inexpensive, large-scale hybrid seed multiplication. The economic benefits of the application of apomixis to agriculture are immense and have been described elsewhere (Hanna, 1995; Koltunow *et al.*, 1995a; Vielle-Calzada *et al.*, 1996; Ramulu *et al.*, 1999). Apomixis is not evident in current major cereal, grain, and vegetable crops. It is primarily found in non-agronomic plants, and only a few of these are sufficiently related to cross with crops for introgression of the trait by hybridization.

Apomictic species are being used as research models for the identification and isolation of the genes conferring apomixis and the downstream pathways they control, to provide tools for the development of apomixis as a technology in hybrid seed breeding. Few species are obligate apomicts; most are facultative apomicts, meaning that they retain the capacity to form seeds via the sexual pathway to varying degrees (Asker and Jerling, 1992). The analysis of evolved apomictic species with various mechanisms is informative for understanding molecular, biochemical, and ecological factors associated with high rates of apomictic seed set. In addition to elucidating the molecular basis for apomixis in evolved species, a parallel research approach is aimed at identifying mutations in genes or novel alleles that modify aspects of sexual seed development giving apomixis pathway–like phenotypes, and combining them to synthesize a pathway resembling apomixis that enables formation of seeds containing embryos with a maternal genotype (Chaudhury and Peacock, 1993).

In this chapter, we review progress in the understanding of the developmental biology of apomixis and its evolution, distribution, genetic regulation, and progress toward isolating apomixis genes from functional apomictic species. We describe genes involved in sexual reproduction that, when mutated, stimulate apomixis-like phenotypes, and we highlight more recent success in the generation of asexual seeds in *Arabidopsis* following the combination of specific mutants. We also consider some issues for the installation of apomixis in hybrid crops in light of current knowledge.

Biology of Apomixis in Natural Systems

Overview of Sexual Seed Formation Pathway

Apomixis is broadly divided into two types: gametophytic and sporophytic. Both types omit steps found in the sexual pathway. The diversity of apomictic pathways has been covered in many reviews (Nogler, 1984a; Koltunow, 1993; Koltunow and Grossniklaus, 2003). The distinctions between sporophytic and gametophytic apomixis are best understood if compared with the sequence of events during sexual seed formation (Figure 5.1). The sexual mode of seed formation is retained in most apomicts owing to their facultative nature. In angiosperms, sexual reproduction is characterized by an alternation of generations life cycle involving the transition between a diploid (2n) sporophytic generation and a haploid (n) gametophytic generation that gives rise to a diploid sporophyte following double fertilization. Diploid (2n) male and female gamete precursor cells termed microspore and megaspore mother cells undergo meiosis in the anther and ovule to produce haploid (n) female megaspores and male microspores. They subsequently undergo mitosis to give rise to multicellular gametophytes, the male pollen grain and female embryo sac containing the sperm and egg cells. Figure 5.1*a* shows the events of female gametophyte development in the *Polygonum*-type embryo sac, the most common type formed in angiosperms. A single megaspore mother cell differentiates and undergoes megasporogenesis, which involves the events of meiotic reduction to produce four haploid megaspores, and subsequent megaspore selection where three of the four megaspores formed degenerate. The surviving megaspore undergoes megagametogenesis, which involves three rounds of mitosis without cytokinesis generating a syncytium of eight nuclei. Subsequent cellularization

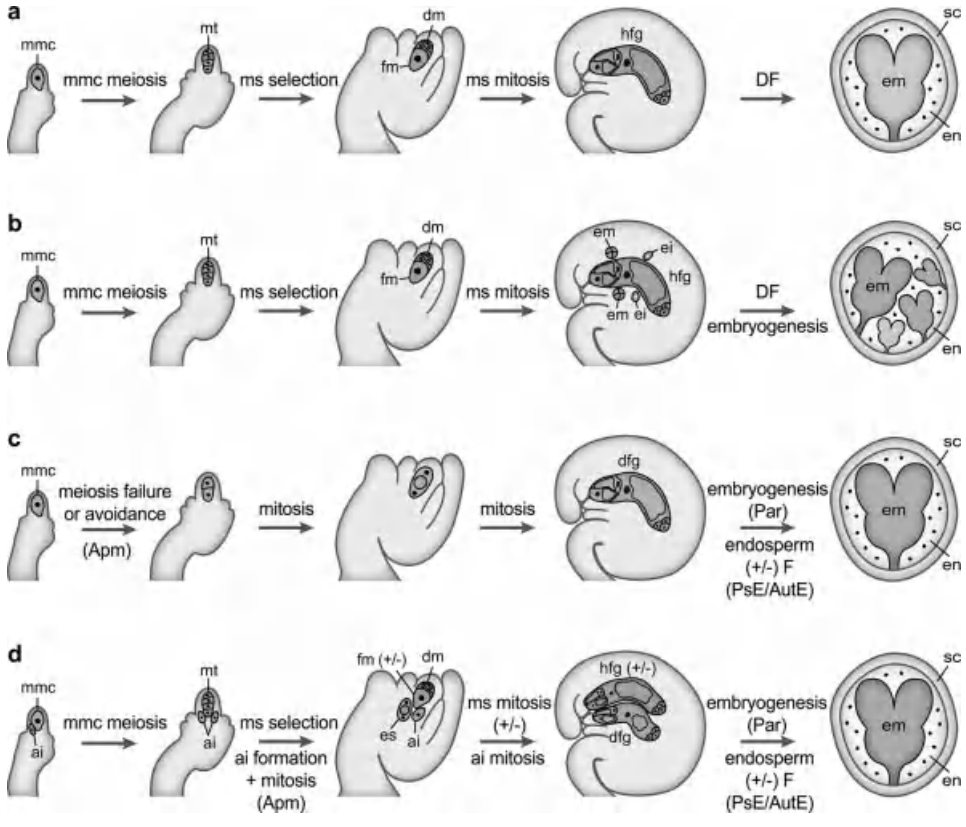

Figure 5.1 Seed development in sexual and apomictic species. *a,* Events of sexual seed formation. *b,* Events of sporophytic apomixis. *c,* Events of diplosporous apomixis and, *d,* aposporous apomixis, where endosperm formation may or may not require fertilization. In the case of apospory, haploid female gametophytes may coexist with the aposporous gametophyte, or degeneration of the haploid gametophyte may occur during the events of aposporous gametophyte development. See text for further details. ai, aposporous initial cells; Apm, apomeiosis; AutE; autonomous endosperm formation; DF, double fertilization; dfg, diploid female gametophyte; dm, degenerating megaspores; ei, embryo initial cell; em, embryo; en, endosperm; F, fertilization; fm, functional megaspore; hfg, haploid female gametophyte; mmc, megaspore mother cell; ms, megaspore; mt, meiotic tetrad; Par, parthenogenesis; PsE, pseudogamous endosperm formation which requires fertilization; sc, seed coat; (+/−), present or absent. (Modified from Drews and Koltunow, 2011.) (For color detail, see color plate section.)

and cell differentiation events result in the formation of a mature female gametophyte containing an egg cell, two synergids, three antipodals, and a central cell with two haploid nuclei.

Subsequent seed formation requires double fertilization. A haploid sperm cell fuses with the haploid egg nucleus to generate a diploid zygote, with a 1 maternal (m):1 paternal (p) genome ratio, which differentiates into the diploid embryo. Fusion of a second sperm cell with the two central cell nuclei generates a triploid endosperm precursor cell with a 2m:1p genome ratio that proliferates to form nutritive endosperm. In cereals such as maize, the 2m:1p genome ratio is critical for endosperm viability (Koltunow and Grossniklaus, 2003). In many grasses and cereals, the antipodals proliferate after fertilization, whereas they degenerate before or during fertilization in other species. The ovule tissues surrounding the fertilized female gametophyte develop into the seed coat (Maheshwari, 1950; Willemse and van Went, 1984; Drews and Koltunow, 2011).

Sporophytic Apomixis

Sporophytic apomixis involves the direct formation of one or more embryos from diploid ovule cells, termed embryo initial (ei) cells, which differentiate adjacent to a developing female gametophyte (Figure 5.1*b*). It is common in mango and *Citrus* and is often termed adventitious embryony. Analyses in *Citrus* show that ei cells are specified early in ovule development, during megaspore mitosis. If the adjacent haploid embryo sac is fertilized, the zygotic embryo may or may not survive the competition for resources associated with multiple embryo growth in the viable polyembryonic seeds formed. If the embryo sac is not fertilized, multiple embryos derived from ei cells apparently grow by using nutrient derived from digestion of the massive nucellus in the ovule, and they arrest at the globular and heart stages of embryogenesis. Tissue culture can be used to rescue these embryos, which develop into viable seedlings (Koltunow *et al.*, 1995b). Polyembryonic seeds are commonly used to generate rootstocks for citrus propagation (Kepiro and Roose, 2010). Sporophytic apomixis is of minor focus in this chapter. It has not been extensively examined, although independent genetic studies indicate dominant control and simple inheritance of polyembryony in citrus (Garcia *et al.*, 1999; Nakano *et al.*, 2008a, 2008b; Kepiro and Roose, 2010).

Gametophytic Apomixis

Gametophytic apomixis results in the formation of a diploid or chromosomally unreduced female gametophyte because meiosis is bypassed by processes grouped under the term apomeiosis (Apm) (Figure 5.1*c* and *d*). An egg cell differentiates in the unreduced embryo sac and develops into an embryo in the absence of fertilization in a process termed parthenogenesis (Par). Endosperm formation in gametophytic apomicts may be fertilization independent (autonomous endosperm [AutE]) or may require fertilization (pseudogamous endosperm [PsE]). Autonomous endosperm formation is rare in apomicts and is predominantly found in the Asteraceae. Table 5.1 provides examples of numerous monocot and eudicot gametophytic apomicts and their modes of unreduced embryo sac and endosperm formation.

Apomeiosis occurs by two major routes, diplospory (Dip) (Figure 5.1*c*) and apospory (Apo) (Figure 5.1*d*), distinguished by the origin of the precursor cell giving rise to the unreduced embryo sac. In diplospory, the megaspore mother cell remains unreduced because either meiosis never initiates (mitotic diplospory, *Antennaria*-type) or it fails (meiotic diplospory), and the cell undergoes restitution at an early stage. Recombination has been observed in some forms of meiotic diplospory (van Baarlen *et al.*, 2000). Mitotic divisions ensue to generate an embryo sac (Figure 5.1*c*). In apospory, the megaspore mother cell differentiates, and sexual female gametophyte development initiates. Additional cells, termed aposporous initial (ai) cells, differentiate in close proximity to cells undergoing sexual female gametophyte development. One or more ai cells undergo mitosis to produce unreduced embryo sacs. The timing and frequency of ai cell appearance in relation to the events of the sexual pathway vary in different species. Typically, ai cells appear soon after megaspore mother cell enlargement or during megaspore tetrad formation (Figure 5.1*d*). In aposporous *Pennisetum* and *Hieracium* species, the sexual pathway terminates soon after ai cells begin to enlarge or undergo mitosis (Snyder, 1955; Peel *et al.*, 1997; Koltunow *et al.*, 1998, 2000, 2011a), whereas in other species both reduced and aposporous embryo sacs may coexist in ovules (Figure 5.1*d*).

Many unreduced embryo sacs contain eight nuclei before cellularization occurs and structurally resemble the sexual *Polygonum*-type embryo sac described earlier. These "eight-nucleate" unreduced embryo sacs are termed *Hieracium*-type in the apomixis literature. The *Panicum*-type, "four

Table 5.1 Features of Gametophytic Apomictic Model Systems under Study

Species	Apomixis type[a]	Traits Mapped[b]	Loci	References for Inheritance and Molecular Markers	Chromosomal Location	Transformable	Genome Sequence/No. Nucleotide Sequences in GenBank[c]
Taraxacum officinale (e)[d]	Dip AutE	Apm/Par/AutE	2–3	Tas and Van Dijk, 1999; Van Dijk *et al.*, 1999; Vijverberg *et al.*, 2004, 2010	nd[e]	nd	No/264 nucleotide; 41296 EST; 0 GSS; 0 SRA
Tripsacum dactyloides (m)	Dip PsE	Apm	1	Leblanc *et al.*, 1995; Grimanelli *et al.*, 1998b	nd	nd	No/548 nucleotide; 5 EST; 48 GSS; 0 SRA
Erigeron annuus (e)	Dip AutE	Apm/Par-AutE	2	Noyes and Rieseberg, 2000; Noyes *et al.*, 2007	nd	nd	No/51 nucleotide; 0 EST; 0 GSS; 0 SRA
Boechera divaricarpa (e)	Dip PsE	Clonal progeny	nd	Schranz *et al.*, 2006	nd	nd	*B. holboellii* pending/597 nucleotide *for B. divaricarpa*
Pennisetum squamulatum (m)	Apo (4-nucl)[f] PsE	Apm	1	Ozias-Akins *et al.*, 1993, 1998; Goel *et al.*, 2006; Huo *et al.*, 2009; Zeng *et al.*, 2011	Apm: telomeric (Akiyama *et al.*, 2004)	nd[g]	No/84 nucleotide; 0 EST; 2389 GSS; SRA Accn. SRX047367
Cenchrus ciliaris (m)	Apo (4-nucl) PsE	Apm or Apm-Par	1	Sherwood *et al.*, 1994; Gustine *et al.*, 1997; Roche *et al.*, 1999; Jessup *et al.*, 2002; Dwivedi *et al.*, 2007	Apm: interstitial/ pericentromeric (Akiyama *et al.*, 2005)	No stable	No/47 nucleotide; 21733 EST; 2145 GSS; 0 SRA
Paspalum simplex (m)	Apo (8-nucl)[h] PsE	Apm-Par	1	Pupilli *et al.*, 2001; Labombarda *et al.*, 2002	Distal (Calderini *et al.*, 2006)	nd	No/7 nucleotide; 0 EST; 0 GSS; 0 SRA
Hieracium spp (e)	Apo AutE	Par; Apm/Par-AutE	1 (Par); 2 (Apm/Par-AutE)	Bicknell *et al.*, 2000; Catanach *et al.*, 2006; Koltunow *et al.*, 2011a; Okada *et al.*, 2011	Apm (*LOA*) subtelomeric (Okada *et al.*, 2011)	Yes (Bicknell and Borst, 1994)	No/840 nucleotide; 132 EST; 0 GSS; SRA Accn. SRX082257
Hypericum perforatum (e)	Apo PsE	Apm/Par	2	Schallau *et al.*, 2010	nd	Yes (Franklin *et al.*, 2007)	No/63 nucleotide; 3 EST; 0 GSS; 22 SRA[i]

(*continued*)

Table 5.1 (*Continued*)

Species	Apomixis type[a]	Traits Mapped[b]	Loci	References for Inheritance and Molecular Markers	Chromosomal Location	Transformable	Genome Sequence/No. Nucleotide Sequences in GenBank[c]
Brachiaria brizantha (m)	Apo (4-nucl) PsE	Apm	1	do Valle and Savidan, 1996; Pessino *et al.*, 1997, 1998	nd	No stable	No/16 nucleotide; 2203 EST; 0 GSS; 0 SRA
Brachiaria humidicola (m)	Apo (4-nucl) PsE	Apm	1	Zorzatto *et al.*, 2010	nd		No/31 nucleotide; 0 EST; 0 GSS; 0 SRA
Panicum maximum (m)	Apo (4-nucl) PsE	Apm	1	Hanna *et al.*, 1973; Savidan, 1980; Ebina *et al.*, 2005	nd	No stably transformed plants[g]	No[j]/116 nucleotide; 196 EST; 0 GSS; 0 SRA
Paspalum notatum (m)	Apo (4-nucl) PsE	Apm	1	Martinez *et al.*, 2001, 2003; Stein *et al.*, 2007; Acuna *et al.*, 2009, 2011	nd	Yes (Smith *et al.*, 2002; Altpeter and James, 2005)	No/80 nucleotide; 0 EST; 0 GSS; 0 SRA
Poa pratensis (m)	Apo (8-nucl) PsE	Par; Apm/Par; Apm/Par	1 (Par); 2 (Apm/Par); 5 (Apm/Par)	Barcaccia *et al.*, 1998; Albertini *et al.*, 2001; Matzk *et al.*, 2005	nd	Yes (Ha *et al.*, 2001; Gao *et al.*, 2006)	No/239 nucleotide; 0 EST; 0 GSS; 0 SRA
Ranunculus auricomus (e)	Apo PsE	Apm/Par	1–2	Nogler, 1984b, 1995	nd	nd	No/8 nucleotide; 0 EST; 0 GSS; 0 SRA

[a]The mode of gametophytic apomixis in each species is denoted by the mode of unreduced embryo sac formation, either diplosporous (Dip) or aposporous (Apo). All form unreduced embryo sacs and undergo autonomous embryo development from unreduced eggs. The mode of endosperm formation is autonomous (AutE) or pseudogamous (PsE), which requires fertilization.

[b]Apm, apomeiosis; Par, parthenogenesis; AutE, autonomous endosperm.

[c]http://www.ncbi.nlm.nih.gov/ entries as of 06 Dec 2011. EST, expressed sequence tag; GSS, genome survey sequence; SRA, sequence read archive.

[d](e), eudicot; (m), monocot.

[e]nd, not determined (no physical data).

[f]Unreduced 4-nucleate *Panicum*-type aposporous embryo sacs are formed.

[g]Sexual *Pennisetum glaucum* has been transformed (Goldman *et al.*, 2003); sexual *Panicum virgatum* (switchgrass) has been transformed (Somleva *et al.*, 2002; Li and Qu, 2011).

[h]Unreduced 8-nucleate aposporous embryo sacs are formed.

[i]*Hypericum perforatum* SRA accessions: SRX062061-SRX062062, SRX062064-SRX062066, SRX062068-SRX062069, SRX062072-SRX062073, SRX062133-SRX062141, SRX062143-SRX062146.

[j]There is no genome sequence for *Panicum maximum,* although switchgrass (*P. virgatum*) and a diploid relative (*P. hallii*) are being sequenced by DOE-JGI; most *Panicum* sequences in GenBank (>700,000 ESTs; ~200,000 GSS; 114 SRA) are from switchgrass.

nucleate" embryo sac found in many aposporous grasses is a notable exception. Only two rounds of mitosis occur, generating four nuclei that typically form an egg, two synergids, and a uninucleate central cell after cellularization. This diploid, uninucleate central cell in four-nucleate *Panicum*-type embryo sacs may be an adaptation to maintain a viable 2m:1p genome ratio in the pseudogamous endosperm, following fertilization (Koltunow and Grossniklaus, 2003).

Variations in mature unreduced embryo sac structure have been observed within the same genus. *Paspalum notatum* is characterized by four-nucleate *Panicum*-type aposporous embryo sacs, whereas *P. simplex* forms eight-nucleate aposoporous embryo sacs that are essentially indistinguishable from reduced embryo sacs of the *Polygonum*-type. Cytological analyses in *Hieracium* subgenus *Pilosella* species also indicate that the unreduced eight-nucleate *Hieracium*-type embryo sac is not always the stable outcome in aposporous species described by Rosenberg (1907) when apomixis was discovered in the genus. Abnormality in both the number of mitotic divisions and the way embryo sacs form have been described that lead to variations in embryo sac structure. For example, multiple developing embryo sacs may amalgamate to form a single structure of varying nuclear content in some aposporous *Hieracium* species (Koltunow *et al.*, 1998, 2000, 2011a).

Phylogenetic and Geographical Distribution of Apomixis

Perhaps the most recent comprehensive analysis of the phylogenetic distribution of apomixis was undertaken by Carman (1997). Of the 460 angiosperm families according to Judd *et al.* (1994), 33 contain gametophytic apomicts and are distributed across the more primitive lineages of Ranunculales and Piperales to the more advanced Poales and Asterales (Carman, 1997). More genera in the Poaceae (grass) and Asteraceae (sunflower) families contain apomicts than those of other plant families, with at least 38 and 35 genera (Ozias-Akins, 2006). Asteraceae and Poaceae are the first and fourth largest plant families in terms of number of genera and species, separated by Orchidaceae (orchids) and Fabaceae (legumes) (www.mobot.org). Gametophytic apomicts have not been reported in the Fabaceae even though it is a member of the rosids where other families demonstrating apomixis occur.

Apomixis is not the prevailing reproductive mode in evolution (Rollins, 1967). Given its broad distribution among angiosperms, yet infrequent occurrence, gametophytic apomixis is regarded as polyphyletic, and apomicts that either are more recently derived or confer some adaptive advantage are those that are extant. Asexual reproduction may be favored in geographically marginal habitats where sexual reproduction has a low rate of success (Silvertown, 2008), according to the survivalist "escape from sterility" yet "escape into a blind alley of evolution" theories described several decades ago (Darlington, 1939). However, geographical apomixis cannot be explained by a single predominant evolutionary force such as hybridization, polyploidy, male function, niche differentiation, or biotic interactions (Hoerandl, 2006). The "twiggy" phylogenetic distribution of asexuals, usually interpreted as an evolutionary dead end pattern, can also be predicted by three neutral models, only one involving extinction (Schwander and Crespi, 2009). The first posits that transition from sexual to asexual reproduction is rare, the second that origin and neutral loss are in equilibrium (Janko *et al.*, 2008), and the third that hybrid origin of asexuals is more frequent in high latitudes where extinction rates also are higher. These models were tested empirically with data only from animals but were proposed as alternatives to selective models used to explain "twigginess" of both animal and plant asexuals (i.e., mutation accumulation and ecological adaptation). Most investigations of distribution do not take into account the facultativeness of apomixis where the capacity for sexual reproduction within a clone enables recombination and gene flow to varying degrees, a phenomenon

well documented in *Ranunculus*, *Taraxacum* (Hoerandl and Paun, 2007), and *Hieracium* (Fehrer *et al.*, 2007).

Part of the difficulty in phylogenetic comparison of sexuals and asexuals for testing of these models in plants originates with defining the species; asexuals often are considered members of polyploid agamic complexes (de Wet, 1968; Hoerandl, 1998). To study asexuals empirically compared with sexuals and to avoid the confounding effects of polyploidy and changes in ploidy invariably encountered among apomicts, Johnson *et al.* (2010) took advantage of a reproductive anomaly in evening primrose (*Oenothera*), permanent translocation heterozygosity or PTH, that parallels apomixis in its clonal reproductive outcome but occurs in a diploid organism. Diversification rates in asexual lineages were significantly faster (eightfold) than in sexual lineages where recombination and segregation can be homogenizing forces (Johnson *et al.*, 2011). This study provides evidence that asexual lineages are not evolutionary dead ends despite a constraint for adaptive evolution.

Inheritance of Apomixis

As discussed previously, sporophytic and gametophytic apomixis can be broken down into three developmental components. These include the avoidance of meiosis (Apm) to generate an unreduced embryo sac containing a cell capable of fertilization-independent embryogenesis (Par) and the generation of viable endosperm, which may occur by fertilization-dependent or fertilization-independent means. One to five dominant genetic loci have been proposed to control one or more of the three aforementioned components of apomixis in different species. However, the apparent simple Mendelian inheritance observed in many of these species (Table 5.1) is, most likely, an artifact of genes residing in a region of the genome suppressed for recombination (Ozias-Akins *et al.*, 1998; Roche *et al.*, 2001a; Pupilli *et al.*, 2004; Ozias-Akins and van Dijk, 2007). Recombination in some species has led to separation of traits for unreduced embryo sac formation and parthenogenesis, suggesting that at least two genes are required for apomixis. Even in these cases, however, one of the two loci may be in a region of reduced recombination (e.g., parthenogenesis in *Taraxacum* (van Dijk *et al.*, 2009) and diplospory in *Erigeron* (Noyes and Rieseberg, 2000)). Low recombination around apomixis loci may result from local chromosomal rearrangements that become fixed because of the asexual mode of reproduction. These recombination-deficient regions may ensure linkage disequilibrium for coding and regulatory regions that maximize the penetrance of apomixis (Vijverberg *et al.*, 2010).

Of the genetic mapping studies that have been conducted (Ozias-Akins and van Dijk, 2007), the most informative are in monocots *Pennisetum*, *Paspalum*, *Poa*, and *Tripsacum*, all members of the grass family (Poaceae), and in eudicots *Hieracium*, *Erigeron*, and *Taraxacum* (Table 5.1). No in-depth genetic studies have been undertaken on the near-diploid apomictic *Boechera holboellii* that is closely related to the model sexual plant species *Arabidopsis thaliana* and whose genome is presently being sequenced by the DOE Joint Genome Institute.

Simple dominant Mendelian inheritance of apospory or apomixis has been observed in *Pennisetum squamulatum*, *Cenchrus ciliaris*, *Panicum maximum*, *Tripsacum dactyloides*, multiple *Brachiaria* and *Paspalum* species, and *Ranunculus auricomus* where crosses between apomictic males and sexual females have been conducted (Table 5.1). Intraspecific crosses are possible when a sexual polyploid ecotype occurs, or the chromosome number of a sexual diploid has been artificially doubled to make a ploidy compatible cross with an apomictic tetraploid. In other species where no sexual ecotypes have been identified, interspecific crosses have been necessary to study inheritance. Given the heterozygosity of polyploid apomicts, genetic mapping can be conducted in the F_1

generation where sexual and apomictic modes of reproduction segregate 1:1. Significant deviation from 1:1 segregation has been observed most prominently in *Paspalum notatum* and interpreted as selection against male gametes carrying an apomixis allele either because of pleiotropy, incompletely penetrant lethality or a linked deleterious allele (Martinez *et al.*, 2001). Transmission of the *LOA* apomeiosis locus in aposporous *Hieracium* varies among species with exceptionally poor transmission of *LOA*-linked sequences when apomictic *H. caespitosum* (C36) is the pollen donor (Okada *et al.*, 2011). Less extreme segregation distortion has been observed in other *Hieracium* species (Catanach *et al.*, 2006; Okada *et al.*, 2011) and in other apomicts (Ozias-Akins *et al.*, 1998; Grimanelli *et al.*, 1998a, 1998b; Ozias-Akins and van Dijk, 2007). There is also evidence that transmission can vary between male and female gametes when sufficient numbers of reduced female gametes can be analyzed (Roche *et al.*, 2001b).

Although in most grass species, the events of unreduced embryo sac formation and parthenogenesis have not been genetically separated, there are some possible exceptions, most notably in *Poa pratensis* (Albertini *et al.*, 2001). Of 38 hybrids from a sexual × apomict cross, 22 were identified as non-parthenogenetic, and 20 of those showed only sexual development. In the two exceptional cases, aposporous embryo sacs were formed, but no embryos developed after auxin treatment, the typical test for parthenogenesis in *Poa* (Matzk, 1991), suggesting that unreduced embryo sac formation and parthenogenesis were independently controlled.

Kaushal *et al.* (2008) also described an "uncoupling" of apomixis components in *Panicum maximum* based on single seed flow cytometric seed screening (FCSS) (Matzk *et al.*, 2000). Reproductive versatility was demonstrated in some lines, for example, apospory with varying levels of parthenogenesis and occasional autonomous instead of the expected pseudogamous endosperm formation. None of these developmentally variable lines exclusively lacked parthenogenesis (i.e., formed fertilized unreduced eggs) or formed reduced eggs that underwent autonomous embryogenesis (i.e., lacked nonreduction). These results are better interpreted perhaps as possible examples of early failure of parthenogenesis in the case of putative autonomous endosperm detection or incomplete penetrance or expressivity of apomictic components.

Similar observations that occurrence of apomeiosis and parthenogenesis were highly correlated but that parthenogenesis frequency never exceeded apomeiosis frequency were made from FCSS of *Boechera spp*. accessions (Aliyu *et al.*, 2010). In *Boechera*, few controlled crosses to examine inheritance of apomeiosis and parthenogenesis have been made, and they have not supported a simple model of inheritance for apomixis (Schranz *et al.*, 2005, 2006). Many progeny of controlled crosses have been sterile (Schranz *et al.*, 2005), interpreted as lack of transmission of apomixis, and encumber genetic analysis of progeny. Cytological observation of embryo sac development was not conducted in these studies where inferences for genetic control of apomixis were made from assessment of progeny uniformity, fertility, and karyotype analyses. The transmission of apomixis was not associated with a single dominant factor on the heterochromatic (*Het*) chromosome that typically is found in apomicts because triploid progeny derived from a cross of diploid sexual *B. stricta* with diploid *B. divaricarpa* contained the full chromosome complement from the diploid apomictic parent, including the *Het* chromosome, but did not provide evidence for apomictic reproduction on progeny analysis (Schranz *et al.*, 2006). An apomixis factor on *Het* was not completely ruled out given that recombination could have occurred during the formation of the unreduced gametes through meiotic diplospory.

A complex five-locus model was invoked by Matzk *et al.* (2005) to explain variable penetrance of apomixis components in *Poa* that involves apospory initiator and preventer genes, parthenogenesis initiator and preventer genes, and a megaspore development gene. Segregation of apomixis components in progeny of several crosses was interpreted according to the model. Their analyses

confirmed that aposporous plants lacking parthenogenetic capacity can be recovered in *Poa*, but the converse was not found. In these examples, it appears that parthenogenesis is largely dependent on apomeiosis, leaving open the question of control by an independent genetic element or pleiotropy.

Genetic studies in species of the family Asteraceae resolve parthenogenesis and apomeiosis as genetically distinct. Unlinked loci controlling apomeiosis and parthenogenesis have been clearly demonstrated in *Erigeron annuus*, *Taraxacum officinale*, and *Hieracium caespitosum* (reclassified as *H. praealtum*, genotype R35). All of these species form autonomous endosperm (Table 5.1). Genetic mapping of structured populations was used to determine the unlinked nature of these loci in *Erigeron* (Noyes and Rieseberg, 2000) and *Taraxacum* (Tas and van Dijk, 1999; van Dijk and Bakx-Schotman, 2004; van Dijk *et al.*, 1999), whereas mapping of mutations was used in *Hieracium* (Catanach *et al.*, 2006).

In *Taraxacum*, hybrid progeny were recovered at low frequency from a cross of a sexual diploid with apomictic triploids, and ploidy levels ranged from diploid to triploid and tetraploid. All tetraploids and one third of the triploids set seeds after emasculation establishing their apomictic mode of reproduction (Tas and van Dijk, 1999). Of the two thirds "nonapomictic" triploids and diploids, no seeds were set after emasculation. Subsequent analysis determined that diploids were sexual, but some of the "nonapomictic" triploids formed unreduced female gametes that developed only if fertilized indicating that apomeiosis and parthenogenesis were uncoupled in *Taraxacum* (van Dijk *et al.*, 1999). In one of the triploids, endosperm did initiate in the absence of pollination, but the egg cell remained undivided, suggesting that autonomous endosperm formation and parthenogenesis may also be uncoupled (van Dijk *et al.*, 2003).

Further genetic analysis in *Taraxacum* has focused on the *DIP* (diplospory) locus in genetic backgrounds where parthenogenesis is absent, simplifying genetic analysis and demonstrating that *DIP* behaves as a dominant, simplex allele (van Dijk and Bakx-Schotman, 2004; Vijverberg *et al.*, 2004). Yet this apparent simplicity may, as with several grasses, be complicated by duplication and local chromosomal rearrangements, complexity that was introduced to explain recombinants with reduced penetrance of diplospory (Vijverberg *et al.*, 2010).

Although no large-scale repression of recombination has been observed around *DIP* in *Taraxacum*, another diplosporous member of the same plant family, *Erigeron*, has shown recombination reduction associated with diplospory (Noyes and Rieseberg, 2000). As with *Taraxacum*, uncoupling of *DIP* and parthenogenesis allowed the recovery of *DIP*-only genotypes that showed an increase in chromosome number owing to fertilization of the unreduced egg cell (Noyes, 2005). The converse, parthenogenesis of reduced eggs, was not recovered, although functional apomixis was restored in progeny of *DIP*-only genotypes crossed with a female sterile plant that contained all of the molecular markers linked with parthenogenesis (Noyes, 2006). Parthenogenesis was restored more frequently after fertilization with diploid rather than haploid male gametes, although gametophytic versus zygotic lethality was not determined. Three apomicts with restored parthenogenesis did not contain any of the parthenogenesis-linked markers showing that linkage could be broken. No evidence for independent control of parthenogenesis and autonomous endosperm formation has been observed in *Erigeron,* and autonomous seed development was considered possibly due to pleiotropy of a single gene (Noyes *et al.*, 2007).

Mutation mapping in *Hieracium praealtum* and supporting genetic crosses have established that apomeiosis and autonomous seed initiation are controlled by two dominant, independent loci (Catanach *et al.*, 2006; Koltunow *et al.*, 2011a). Deletion of the dominant apomeiosis locus, *LOSS OF APOMEIOSIS* (LOA), recovered *loa* mutants, characterized by their inability to form ai cells. Deletion of the *LOSS OF PARTHENOGENESIS* (*LOP*) locus in *H. praealtum* recovered *LOAlop* mutants that formed unreduced embryo sacs but were unable to undergo autonomous seed

formation. Pollination of *LOAlop* mutants resulted in progeny with increased ploidy level indicating fertilization of unreduced eggs. On analysis of unpollinated *loa* mutants carrying a functional *LOP* locus (*loaLOP*), 50% of seeds (*loaLOP*) developed as a consequence of autonomous embryo and endosperm formation, and progeny had half the chromosome number of the parent. This finding indicated that autonomous seed development occurred from a meiotically reduced egg and that *LOP* was segregating gametophytically in reduced embryo sacs owing to loss of *LOA* function (Koltunow *et al.*, 2011a). The identified *loalop* mutants exhibited a complete reversion to sexual reproduction, confirming that sexual reproduction is intact in the apomict *H. praealtum*, and it is the default reproductive pathway once *LOA* and *LOP* are eliminated from the genome. Further evidence in *Hieracium praealtum* suggests that *LOP* acts pleiotropically to induce autonomous embryo and endosperm development, or, if multiple genes are required, they are tightly linked (Koltunow *et al.*, 2011a).

An aposporous eudicot outside of the sunflower family with *Hieracium*-type unreduced embryo sac development but primarily pseudogamous endosperm formation is *Hypericum perforatum* (Martonfi *et al.*, 1996). *H. perforatum* displays considerable heterogeneity for reproductive outcomes where 11 routes have been observed (Matzk *et al.*, 2001). Seeds derived through apomeiosis without parthenogenesis and parthenogenesis without apomeiosis were identified suggesting that genetic components for these processes could be separated (Matzk *et al.*, 2001; Barcaccia *et al.*, 2006). Genetic analysis confirmed that apospory and parthenogenesis were under independent genetic control with each segregating as dominant, simplex alleles in $4x$ sexual $\times$ $4x$ apomict crosses, although linkage in *cis* at 20 cM was estimated (Schallau *et al.*, 2010).

Apomeiosis and parthenogenesis are likely under independent genetic control, but this independence cannot be easily demonstrated in some species because of their tight linkage. At the present time, there is little evidence for independent genetic control of autonomous endosperm formation. These straightforward conclusions are based on the treatment of apomeiosis, autonomous embryo, and autonomous endosperm development as qualitative traits. Quantitative analysis of these same traits has established that additional loci and genes influence penetrance or expressivity (Matzk *et al.*, 2005).

Genetic Diversity in Natural Apomictic Populations

Most apomicts are polyploid and heterozygous, implicating hybrid origin (Carman, 1997). Polyploidy and heterozygosity function as reservoirs of allelic diversity. True clonality in nature (i.e., obligate apomixis) is rare (Asker and Jerling, 1992), even though that would be the goal of engineered apomixis in crops. Among natural populations, greater than expected variation has been observed (Ellstrand and Roose, 1987; Campbell and Dickinson, 1990; Asker and Jerling, 1992; Hoerandl and Paun, 2007). Simulation of genetic variation in populations for which apomixis has become fixed showed that young populations reflect remnant variability of the sexual genotypes in the same geographic region, except where low pollen fertility affects the probability of fixation, and is strongly dependent on population size (Adolfsson and Bengtsson, 2007). As a population fixed for apomixis ages, genetic variability is expected to decrease because of random genetic drift or selection, although mutation acts to introduce new variation. This model for variation in an asexual population is independent of potential reproductive versatility related to the facultative nature of apomixis.

Facultative apomixis gives rise to varying frequencies of sexually derived progeny including those derived from fertilization of a meiotic egg ($n + n$), along with ploidy changes related to

progeny derived from autonomous development of a meiotic egg (n + 0) and progeny derived from fertilization of an unreduced egg (2n + n) (Asker and Jerling, 1992; Hoerandl and Paun, 2007). These arise as a result of uncoupling of apomeiosis and parthenogenesis either genetically or secondary to incomplete penetrance of apomixis in the ovule (Koltunow *et al.*, 2011a).

Male meiosis in many apomicts is relatively normal leading to viable male gametes that contribute to gene flow between apomicts and sexuals in overlapping geographic regions (van Dijk, 2003). Gene flow is more successful in taxa where interploidy crosses are tolerated, or apomictic and sexual genotypes of the same ploidy coexist. In *Taraxacum,* most apomicts found in nature are triploid, but rare tetraploids also exist in mixed polyploid asexual-diploid sexual populations (Verduijn *et al.*, 2004). Although rare, tetraploids were shown to be the primary source of pollen for the formation of new triploids from sexual, diploid mothers. In the aposporous subgenus *Hieracium Pilosella*, hybridization is common between sexual and apomictic species, and complex population structures with varying ploidy states exist (Fehrer *et al.*, 2007).

Molecular Relationships between Sexual and Apomictic Pathways

Developmentally, gametophytic apomixis appears to omit key steps present in the sexual pathway, including meiosis during gametophyte formation and the requirement for fertilization for embryo and, in some species, endosperm formation. Apomixis and sexuality in gametophytic apomicts are not mutually exclusive developmental programs because they coexist in facultative apomicts. Given these features, it has been proposed that gametophytic apomixis is not a new pathway requiring its own unique infrastructure. Instead, apomixis is thought to result from alterations in control of the sexual pathway, leading to a truncated pathway characterized by the omission of key steps or changes in the timing of events (Koltunow, 1993; Grimanelli *et al.*, 2001; 2003; Koltunow and Grossniklaus, 2003; Tucker *et al.*, 2003). Because most apomicts are polyploid, it has also been proposed that apomixis arose from heterochronic expression of the sexual pathway genes as a consequence of hybridization (Carman, 1997).

Cytological studies in *Tripsacum* showing alteration in timing of developmental events (Grimanelli *et al.*, 2003) and global analysis of gene expression in ovules of *Boechera* (Sharbel *et al.*, 2009, 2010) support the idea of a change in timing of the sexual program in apomixis. Developmental studies in *Hieracium* based on examining marker gene expression have provided support at the molecular level to the view that apomixis results from deregulated expression of the sexual program (Tucker *et al.*, 2003). Marker genes that are expressed in the female gametophyte, in the embryo, and during endosperm development show a similar expression pattern between sexual and asexual *Hieracium*; however, an early marker of the megaspore mother cell (MMC) is not expressed in aposporous initial cells (Tucker *et al.*, 2003). These findings are consistent with the view that apospory in *Hieracium* results in the initiation of gametogenesis directly from a somatic cell, bypassing the normal sequence of MMC specification, meiosis, and gametogenesis that is followed in the sexual pathway. Transcriptome analyses of *Hieracium* aposporous initial cells captured by laser microdissection should provide further information on gene expression programs in this enlarging cell (Koltunow *et al.*, 2011b).

The observation that deletion of *LOA*, the apomeiosis locus, and *LOP*, the locus required for autonomous seed initiation in aposporous *Hieracium praealtum*, results in a reversion to sexual reproduction indicates these loci are not essential for sexual reproduction and further supports their likely involvement in deregulating the default sexual program (Koltunow *et al.*, 2011a). Comparative

transcriptional analyses of the changes in gene expression programs in aposporous *Hieracium* and derived mutants may provide information on the downstream processes influenced by the *LOA* and *LOP* apomixis loci (Koltunow *et al.*, 2011b).

The initiation of the sexual program in aposporous *Hieracium* is required to activate *LOA* function, which enables aposporous initial cell formation. Prevention of MMC meiosis by transgenic ablation, using a cytotoxic marker targeted to the enlarging MMC, inhibits aposporous initial cell formation in *H. piloselloides* (Koltunow *et al.*, 2011a). This provides initial evidence for signaling between sexual and apomictic pathways in *Hieracium*.

The locus controlling apomixis in facultative apomictic maize-*Tripsacum* hybrids containing one *Tripsacum* and two maize genomes behaves differently according to context (Leblanc *et al.*, 2009). When present in unreduced female gametes, it is functional, but when present in reduced gametes, it is nonfunctional and gives rise to progeny that reproduce sexually. These results suggest that the *Tripsacum* apomixis control region may interact with other *Tripsacum*-specific genes or alleles to confer apomixis, which is a barrier to transmission of the trait to sexual maize. The absence of these *Tripsacum*-specific genes in reduced gametes, together with apomixis, and the presence of a sexual phenotype (Leblanc *et al.*, 2009) also suggest that apomixis in diplosporous *Tripsacum* may be imposed on sexuality as found for aposporous *Hieracium* so that loss of apomixis results in reversion to sexuality.

Given the facultative nature of apomixis and outcomes of genetic analyses in other apomictic species, discussed previously, the default reproductive mode is also likely to be sexual reproduction in species other than *Hieracium* and *Tripsacum*. The efficiency of apomictic seed set in facultative apomicts is likely to be dependent on the degree of dominance and the penetrance of the apomictic pathway over the sexual, and potential signaling between the two pathways that may lead to the demise of sexual gametophyte formation in some species.

Features of Chromosomes Carrying Apomixis Loci and Implications for Regulation of Apomixis

Relationships between chromosome structure and the genetic control of apomixis have been proposed in numerous studies. The *DIP* locus of *Taraxacum* is thought to be located on a satellite chromosome (van Dijk and Bakx-Schotman, 2004). All diplosporous *Boechera* apomicts studied contain one or two aberrant chromosomes, *Het* and *Del* (Kantama *et al.*, 2007), although *Het* does not carry a single dominant locus for apomixis (Schranz *et al.*, 2006), and *Del* is not always present. Association of diplospory loci with chromosomal features in these species has not been confirmed.

The chromosome carrying the apomeiotic *LOA* locus in three *Hieracium* subgenus *Pilosella* species shares features with the Apospory Specific Genomic Region (ASGR = Apm + Par) conferring aposporous apomixis in *Pennisetum squamulatum* (Akiyama *et al.*, 2004; Okada *et al.*, 2011). Both are located at the distal subtelomeric end of an individual chromosome in a hemizygous chromosomal region containing repetitive sequences (Table 5.1). The AGSR of aposporous *C. ciliaris*, a *Pennisetum* relative, is pericentromeric in location and also surrounded by complex repeats (Akiyama *et al.*, 2005). This repeat structure is not universally detected at the ASGR of all 12 species of apomictic *Pennisetum* examined (Akiyama *et al.*, 2011). By contrast, the apospory locus in *Paspalum notatum*, a member of the same tribe of the Poaceae family, also has a hemizygous chromosomal location, but there is currently no association of the region with complex repeats (Calderini *et al.*, 2006). Similarly, *LOA*-specific linked markers and associated chromosomal structures are not conserved in aposporous *Hieracium aurantiacum*, which is found in a different haplotype network

group to the three other species where chromosomal features are conserved (Okada *et al.*, 2011). This may reflect polyphyletic evolution of the apospory locus within the grass family as well as subgenus *Pilosella*.

Partial sequencing of genomic DNA at the ASGR (Conner *et al.*, 2008) in *Pennisetum* and *Cenchrus* and DNA at the *LOA* locus in *H. praealtum* tightly linked to apospory shows the presence of abundant Ty-copia and Ty-gypsy transposons in addition to complex locus associated repeats that show no obvious sequence conservation (Okada *et al.*, 2011). These monocot and eudicot species diverged 42–47 million years ago (Barreda *et al.*, 2010), and comparisons of sequences at apomixis loci indicate the ASGR and *LOA* loci do not share a common ancestral origin. Instead, the sequence comparisons indicate convergent evolution of these repeat-rich hemizygous chromosomal regions containing apospory loci.

Current sequence information indicates these apomixis associated genomic regions are gene poor. The prediction is that genes present in these repetitive regions would be under strong selection, presumably with an important functional role, to have survived invasion by transposons and other structural chromosome rearrangements. The hemizygous chromosomal repeat-rich apomeiosis loci would be predicted to be suppressed for recombination, which is the case for *Pennisetum* (Goel *et al.*, 2006). Some limited recombination occurs at the *LOA* locus in *Hieracium praealtum* (Okada *et al.*, 2011) and tetraploid *H. piloselloides* (unpublished), which may facilitate identification of the critical sequences associated with apospory.

The conservation of chromosomal structures in these divergent monocot and eudicot species also suggests that such complex repeat regions associated with these loci may be required for function and maintenance of the apomixis trait. Epigenetic pathways may influence gene expression at apomixis loci surrounded by transposons and complex repeat structures because such regions are often targets of small RNAs that induce RNA-directed DNA methylation that suppresses or alters gene expression (Martienssen *et al.*, 2004; Vaucheret, 2008). Disruptions in epigenetic small RNA and DNA methylation pathways in sexual *Arabidopsis* and maize have led to gametophytic apomixis-like phenotypes in these sexual plants (discussed subsequently). There is currently no direct evidence for a role or the involvement of these pathways in natural apomicts.

Observations of apomixis mutants in *Hieracium* where deletions of *LOA* and *LOP* loci lead to a reversion to sexual reproduction implies that it is unlikely that these loci contain genes essential for the progression of the sexual pathway. These apomixis loci might contain genes that interfere with genetic and epigenetic programs (described subsequently) that function to restrict the numbers of female gamete precursor cells to a single cell and regulate the orderly progression of meiosis and double fertilization in the sexual pathway.

Genes Associated with Apomixis

The genes responsible for the manifestation of apomixis have not yet been identified. The genetic analysis of apomixis, development of markers, partial sequencing of genomic regions containing apomixis loci, and analysis of genes differentially expressed in apomictic versus sexual plants have identified numerous candidate “apomixis” genes. Some of these have functional counterparts in sexual reproduction (discussed subsequently).

The partially sequenced *P. squamulatum* ASGR contains a *BABYBOOM* (BBM)-like gene (Conner *et al.*, 2008). *BBM* was identified in *Arabidopsis* and is an AP2-domain transcription factor. Over-expression in *Arabidopsis* leads to the development of embryos and cotyledons from vegetative tissues (Boutilier *et al.*, 2002). Differential screening of sexual and apomictic ovules in

Poa pratensis has identified a *SOMATIC EMBRYOGENESIS RECEPTOR (SERK)-LIKE KINASE, PpSERK*, and *APOSTART. PpSERK* is thought to be the switch that channels embryo sac development and embryo induction. *APOSTART*, a gene with homology to the *Arabidopsis* lipid-binding START-domain containing protein, is thought to be involved in meiosis and programmed cell death (Albertini *et al.*, 2005). Analysis of the *Hypericum* apospory locus (*HAPPY*) has identified a CAPS marker cosegregating with apospory but not with parthenogenesis, which is independently controlled in *Hypericum* (Schallau *et al.*, 2010). The marker is part of a gene showing high similarity to the ARIADNE (ARI) family of proteins, a class of ring finger proteins that putatively function as E3 ubiquitin protein ligases and has high similarity to *Arabidopsis ARI7*. Comparison of sexual and apomictic alleles showed that the allele identified by the CAPS marker in aposporous plants was truncated and surrounded by copia-like transposons and pseudogenes. The tetraploid apomict also carries three other intact alleles, whereas the sexual plant has four intact copies of the gene (Schallau, 2010). The role of all of these genes in apomixis is unclear. In some cases, progress to clarify their functional roles is hampered by the inability to transform sexual and apomictic species under study (Table 5.1), and surrogate sexual models are being used.

Mutations in members of the *Arabidopsis* FERTILIZATION INDEPENDENT SEED (FIS) complex initiate endosperm proliferation in the absence of fertilization (see later) (Rodrigues *et al.*, 2010a). *Hieracium* orthologues of two *Arabidopsis FIS*-complex genes (*FIE* and *MSI1*), whose functions are elaborated on further subsequently, are not linked to the autonomous seed initiation (*LOP*) locus in *Hieracium praealtum* (Rodrigues *et al.*, 2010b; Koltunow *et al.*, 2011a, 2011b). Downregulation of *Hieracium FIE* (*HFIE*) does not lead to autonomous seed formation in sexual *Hieracium*. However, *HFIE* function is required for the formation of viable fertilization-dependent embryo development in sexual *Hieracium* and for viable autonomous embryo formation in apomictic *Hieracium* (Rodrigues *et al.*, 2008).

Transferring Apomixis to Sexual Plants: Clues from Apomicts

To what extent does the evolution of apomixis in natural systems affect attempts to transfer apomixis or engineer apomixis in crops for which true-breeding hybrids provide a performance advantage? Transfer has been unsuccessful where traditional breeding has taken advantage of crop species with close apomictic relatives (Savidan, 2000). In the case of *Tripsacum*, we discussed previously evidence suggesting the presence of barriers in the transfer of apomixis to maize (Leblanc *et al.*, 2009). The greatest progress to date has been made with introgression of the trait into pearl millet where a single alien chromosome persists in the most advanced apomictic lines (Singh *et al.*, 2010; Zeng *et al.*, 2011).

In retrospect, the slow progress can be explained by the necessity to work at the polyploid level and the genetic consequence of chromosomal repatterning that has occurred in the absence of recombination owing to apomeiosis. Similarly, segregation distortion against apomixis through male (Martinez *et al.*, 2001; Nogler, 1984b; Ozias-Akins *et al.*, 1998) or female (Roche *et al.*, 2001b) gametes may be due to linked deleterious alleles. These properties discourage the use of natural apomicts to develop diploid apomictic crops through hybridization.

If the identified apomixis genes prove nonfunctional in sexual plants because they require additional species-specific factors, strategies for the transfer of apomixis to sexual species may require identification and analysis of the downstream target genes that are regulated by the apomixis control region. Did polyploidy (through hybridization) play a central role in the origin of apomixis, or did it arise as a by-product of enhanced unreduced gamete formation? Will polyploidy be required for

apomixis to function? Derived apomictic polyhaploids (n + 0 progeny) suggest that the latter is not the case even though polyhaploids usually are less fit presumably because of the unmasking of deleterious mutations from the genome of the maternal apomict (Dujardin and Hanna, 1986; Leblanc *et al.*, 1996; Bicknell, 1997).

Synthetic Approach to Building Apomixis

The idea that apomixis results from temporal and spatial alterations in the action of the sexual program suggests the possibility that variant alleles of genes that act in the normal sexual pathway can result in components of apomixis, and that it ought to be possible to identify such genes by mutational analysis in sexual plants (Chaudhury and Peacock, 1993). By identifying and combining the components, it should also be possible to build apomixis in a sexual plant. Molecular genetic analysis in model sexual plants (mainly *Arabidopsis* and rice) has provided support for the validity of this approach. A selected set of genes that are relevant in the context of apomixis is presented in Table 5.2.

Specification of MMC

Analysis of genes controlling sporogenous cell identity and fate in *Arabidopsis*, rice, and maize has identified genes involved in specification of the MMC. Mutations in *Arabidopsis SPOROCYTELESS/NOZZLE* result in failure to form a MMC and defects in nucellar cell identity (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). Mutations in *Arabidopsis WUSCHEL*, a regulator of stem cell identity in the shoot apical meristem, also result in defects in MMC specification (Gross-Hardt *et al.*, 2002). *WUSCHEL* acts by controlling the expression of *WINDHOSE1* and *WINDHOSE2,* which encode small peptides and act in conjunction with *TORNADO2* in controlling megasporogenesis (Lieber *et al.*, 2011). In addition, the *MULTIPLE ARCHESPORIAL CELLS1* (*MAC1*) gene of maize (Sheridan *et al.*, 1996), and the *MULTIPLE SPOROCYTES1* (*MSP1*) and *TAPETUM DETERMINANT LIKE1a* (*TDL1a*) genes in rice (Nonomura *et al.*, 2003; Zhao *et al.*, 2008) are required to limit the number of MMCs to one per ovule. Loss of gene function results in multiple MMCs indicating that these genes negatively regulate sporogenous cell fate in the ovule. The phenotype bears some relationship to apospory wherein multiple cells in the ovule are capable of initiating gametogenesis, although in apospory this occurs without initiating cells undergoing meiosis. *MSP1* encodes a leucine-rich repeat receptor kinase, and *TLD1a* encodes its ligand. Analogous phenotypes to *msp1* mutants occur in male but not female sporogenous development in the *extra sporogenous cells/excess microsporocytes1* (*exs/ems1*) as well as *tapetum determinant1* (*tpd1*) mutants of *Arabidopsis* (Canales *et al.*, 2002; Zhao *et al.*, 2002; Yang *et al.*, 2003). *EXS/EMS1* and *TPD1* are orthologous to *MSP1* and *TDL1a*.

Loss of *ARGONAUTE9* function as well as other genes in the small RNA pathway, *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) and *SUPPRESSOR OF GENE SILENCING3* (*SGS3*), in *Arabidopsis* has been shown to result in dominant loss of restriction in gametic cell identity in the ovule leading to expression of gametic markers in multiple cells of the nucellus that have not undergone meiosis (Olmedo-Monfil *et al.*, 2010). The implication of these results is that gametic cell fate can be uncoupled from meiosis in *ago9* mutants; however, it is unclear whether some of these cells can go on to form functional apomeiotic gametes. A phenotype similar to *ago9* is observed in mutant alleles of *MNEME* (*MEM*), which encodes a DEAD/DEAH box RNA-helicase that shows increased expression in the MMC (Schmidt *et al.*, 2011). In addition to loss of restriction of gametic identity, *mem* mutants also show defects in female gametogenesis and embryogenesis.

Table 5.2 Selected Mutants and Genes from Sexual Plants that Show Features of Apomixis

Mutant/Gene	Mutant Phenotype	Molecule	Reference
Apomeiosis			
SWI1	Diplospory-like	Meiosis-specific chromatin-associated protein	Ravi *et al.*, 2008
AM1	Meiotic non-reduction	SWI1 homolog	Pawlowski *et al.*, 2009
mac1	Multiple archesporial cells	nd	Sheridan *et al.*, 1996
MSP1	Multiple archesporial cells	Leucine-rich repeat receptor kinase	Nonomura *et al.*, 2003
TDL1a	Multiple archesporial cells	Small extracellular protein	Zhao *et al.*, 2008
MiMe (*Atspo11-1 Atrec8 osd1*)	Diplospory-like	Atspo11-1: double-strand endonuclease; Atrec8: meiosis-specific cohesion; osd1: cell-cycle progression protein	d'Erfurth *et al.*, 2009
MiMe-2 (Atspo11-1 Atrec8 tam1)	Diplospory-like	tam1: CYCLIN A1;2	d'Erfurth *et al.*, 2010
AGO9	Apospory-like	ARGONAUTE protein	Olmedo-Monfil *et al.*, 2010
AGO104	Diplospory-like	ARGONAUTE protein	Singh *et al.*, 2011
RDR6	Apospory-like	RNA-dependent RNA polymerase	Olmedo-Monfil *et al.*, 2010
SGS3	Apospory-like	RNA binding protein	Olmedo-Monfil *et al.*, 2010
DMT102	Diplospory-like	DNA methyltransferase	Garcia-Aguilar *et al.*, 2010
DMT103	Diplospory-like	DNA methyltransferase	Garcia-Aguilar *et al.*, 2010
MEM	Apospory-like	RNA helicase	Schmidt *et al.*, 2011
Parthenogenesis/embryogenesis			
hap	Genome elimination	nd	Hagberg and Hagberg, 1980
IG1	Genome elimination, multiple embryos	LOB domain protein	Evans, 2007
LEC1	Promotes embryogenesis	HAP3 subunit of CCAAT box-binding factor	Lotan *et al.*, 1998
BBM1	Promotes embryogenesis	AP2 domain transcription factor	Boutilier *et al.*, 2002
AtSERK1	Promotes embryogenesis	LRR receptor-like kinase	Hecht *et al.*, 2001
MSI1	Parthenogenesis	WD40 domain protein; part of PRC2 Polycomb complex	Guitton and Berger, 2005
Autonomous endosperm			
MEA	Autonomous endosperm	SET domain polycomb protein	Grossniklaus *et al.*, 1998
FIS2	Autonomous endosperm	Part of C2H2 zinc finger protein of Polycomb complex	Luo *et al.*, 1999
FIE	Autonomous endosperm	WD40 domain protein; part of Polycomb complex; EXTRA SEX COMBS (*ESC*) homolog	Ohad *et al.*, 1999
MSI1	Autonomous endosperm	See above	Guitton and Berger, 2005
Endosperm balance			
MET1	Reduction of parent of origin effect on seed size	DNA methyltransferase	Vinkenoog *et al.*, 2001
TTG2	Reduced lethality in interploidy crosses	WRKY transcription factor	Dilkes *et al.*, 2008

Apomeiosis

There are several cases of unreduced gametes arising from sexually derived mutations in plants (Bretagnolle and Thompson, 1995). Unreduced gametes can arise from a range of meiotic abnormalities; however, in most cases the unreduced female gametes do not fully retain the parental genotype and are not truly apomeiotic. It was demonstrated more recently that a mutation in a molecularly characterized gene can lead to apomeiosis for the *dyad* mutant, a hypomorphic allele of the *DYAD/SWITCH1* (*SWI1*) gene of *Arabidopsis* that encodes a truncated protein (Ravi *et al.*, 2008). This finding provided a proof of principle that alteration of a single gene of known molecular identity can lead to a functional component of apomixis including formation of viable seed. *SWI1*, similar to its maize orthologue *AMEIOTIC1* (*AM1*) (Hamant *et al.*, 2006), encodes a meiotic-specific protein required for sister chromatid cohesion and chromosome organization during meiosis. However, the efficiency of seed set in *dyad/swi1* mutants is too low to be directly applied as a synthetic model for diplospory.

An efficient model for diplosporous apomeiosis has been shown to result from the combination of mutations in three genes – *ATSPO11-1*, *ATREC8*, and *OMISSION OF SECOND DIVISION1* (*OSD1)* – that control different aspects of chromosome organization and cell division in meiosis (d'Erfurth *et al.*, 2009). *ATSPO11* encodes a type VI topoisomerase–related protein that is responsible for the formation of recombination-initiating double-strand breaks (Grelon *et al.*, 2001). Chromosomes in the *Atspo11-1* mutant fail to undergo recombination and pairing, remaining as univalents. *ATREC8*, which encodes a meiotic-specific cohesin, is required for co-orientation of sister centromeres and monopolar attachment to the spindle in meiosis I (Bai *et al.*, 1999). In the *Atspo11-1 Atrec8* double mutant, the reductional meiosis I division is replaced by an equational one equivalent to mitosis (Chelysheva *et al.*, 2005). *OSD1* is required for meiosis II, and loss of *OSD1* function leads to omission of the second division of meiosis and formation of unreduced spores. The *Atspo11-1 Atrec8 osd1* triple mutant combination has been termed Mitosis from Meiosis (MiMe) and undergoes a single division that is genetically equivalent to mitosis, resulting in the efficient production of functional apomeiotic female gametes. A similar phenotype is obtained by substituting the *osd1* mutant allele in the above combination with a *tardy asynchronous meiosis* (*tam*) mutant allele (d'Erfurth *et al.*, 2010). *TAM* encodes CYCLIN A1;2, which cooperates with OSD1 in controlling progression through meiosis I and meiosis II.

Arabidopsis ago9, *rdr6*, and *sgs3*, as discussed previously, show a loss in restriction of gametic cell fate. The maize *AGO104* gene is related to *Arabidopsis AGO9*; however, the dominant loss of function *ago104* mutant shows a different phenotype to *ago9* as altered MMC meiosis leads to the formation of functional unreduced female gametes (Singh *et al.*, 2011). The *ago104* mutant phenotype in maize resembles diplosporous apomeiosis, whereas the *Arabidopsis ago9* phenotype resembles apospory. Likewise, the *mel1* mutant of rice (Nonomura *et al.*, 2007), which is in an *Arabidopsis AGO5* related gene, shows a phenotype of meiotic arrest at leptotene. Overall, these findings point to a role for distinct branches of the small RNA pathway in control of early events in megasporogenesis and gametic cell fate specification.

A comparative expression analysis for genes encoding chromatin modifying enzymes between sexual and apomictic ovules of maize-*Tripsacum* hybrids revealed differences for six genes (Garcia-Aguilar *et al.*, 2010). Three of the genes that encode putative DNA methyltransferases (DMT102 and DMT103) and a chromomethylase (CHR106) belong to a class of genes implicated in RNA-directed DNA methylation and are strongly down-regulated in apomictic ovules. *DMT102* and *DMT103* also show down-regulation in autotetraploid maize compared with diploid suggesting that their expression is ploidy dependent, whereas *CHR106* expression was specific to apomictic ovules and

independent of ploidy. A role for DNA methylation in control of meiosis and germ cell formation has been shown in the case of *dmt102* and *dmt103* mutants of maize, which show unreduced male gamete formation as well as the formation of multiple embryo sacs. The origin and ploidy of the extra embryo sac has not been determined (Garcia-Aguilar *et al.*, 2010). These findings suggest that control of DNA methylation is important for normal meiosis and gametogenic cell number in the maize ovule.

Activation of Seed Development

The early mutant screens for apomixis in *Arabidopsis* led to the identification of fertilization-independent initiation of seed development (*FIS*) class genes, which encode members of the Polycomb-related complex (see Chapter 4) (Koltunow and Grossniklaus, 2003). The *fis*-class mutants initiate endosperm development without fertilization with different degrees of expressivity. However, the frequency of embryo initiation by division of the egg cell is lower or does not occur at all for most *fis* mutants. An exception is *multicopy suppressor of ira1* (*msi1*), which gives a high frequency of embryo initiation (90%) (Guitton and Berger, 2005); however, the embryos are inviable.

An additional category of genes has been identified that when over-expressed lead to increased frequency or competence to form somatic embryos either *in planta* or in cell cultures. Several of these genes act as developmental regulators in the normal pathway of embryogenesis. Promoters of somatic embryo formation have been considered as potential candidates for engineering sporophytic apomixis as takes place in adventitious embryony. Members of this class of genes include *LEAFY COTYLEDON1* (*LEC1*) (Lotan *et al.*, 1998), *BABYBOOM1* (*BBM1*) (Boutilier *et al.*, 2002), and *Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*AtSERK1*) (Hecht *et al.*, 2001).

The introduction of apomixis in sexual plants would require addressing the problem of endosperm balance arising out of the change in ploidy of the apomeiotic female gamete. Natural apomicts have adapted themselves in several ways to address the issue of endosperm balance. The evolutionary adaptations range from a change in the fertilization process or in female gametogenesis so as to maintain the 2m:1p genome ratio that is normally required in the endosperm, or a corresponding increase in ploidy of the male gamete, or endosperm and seed development has been made insensitive to m:p ratio as in the case of autonomous apomicts (Koltunow and Grossniklaus, 2003).

Genetically equivalent to parthenogenesis is the phenomenon of uniparental genome elimination. The *INDETERMINATE GAMETOPHYTE1* (*IG1*) gene of maize encodes a lateral organ boundaries (*LOB*) domain protein required for embryo sac and leaf development, and *ig1* mutants exhibit formation of both maternal and paternal haploid embryos (Evans, 2007). Uniparental genome elimination is seen in certain interspecific as well as intraspecific hybrids and results in elimination of the entire set of chromosomes in the embryo from one parent after fertilization. The haploid inducer (*hap*) mutant of barley, which arose as an induced mutation, produces a high frequency of haploids among its progeny (Hagberg and Hagberg, 1980). The act of fertilization triggers seed development, but the genetic contribution from one parent is removed in the developing zygote/embryo after seed development has initiated. The technique of genome elimination has been used in plant breeding for making doubled haploids and for transfer of the genome from one cytoplasm to another. Genome elimination in interspecific crosses has been postulated to be caused by centromere incompatibilities (Bennett *et al.*, 1976). It has been shown more recently that alteration of the centromere-specific histone CENH3 in *Arabidopsis* causes selective elimination of the chromosome set from the mutant parent when crossed to a parent with wild-type CENH3 (Ravi and Chan, 2010). The finding provides a rational and mechanistic basis for engineering haploid inducer lines in sexual plants. Although uniparental genome elimination takes place with partial expressivity, there is potential for increasing

the efficiency of elimination, and the phenomenon is of interest as part of a strategy for synthesis of apomixis.

Synthetic Clonal Seed Formation

The occurrence of apomeiosis in single or combinations of sexually derived mutants of *Arabidopsis* (*dyad* and MiMe) can be combined with *cenh3*-mediated chromosome elimination to produce fully clonal seeds (Figure 5.2). Such a combination of apomeiosis with chromosome elimination has been successfully shown to result in the formation of clonal seeds at a frequency of 35% of viable seeds (Marimuthu *et al.*, 2011). These results demonstrate a proof of principle for being able to

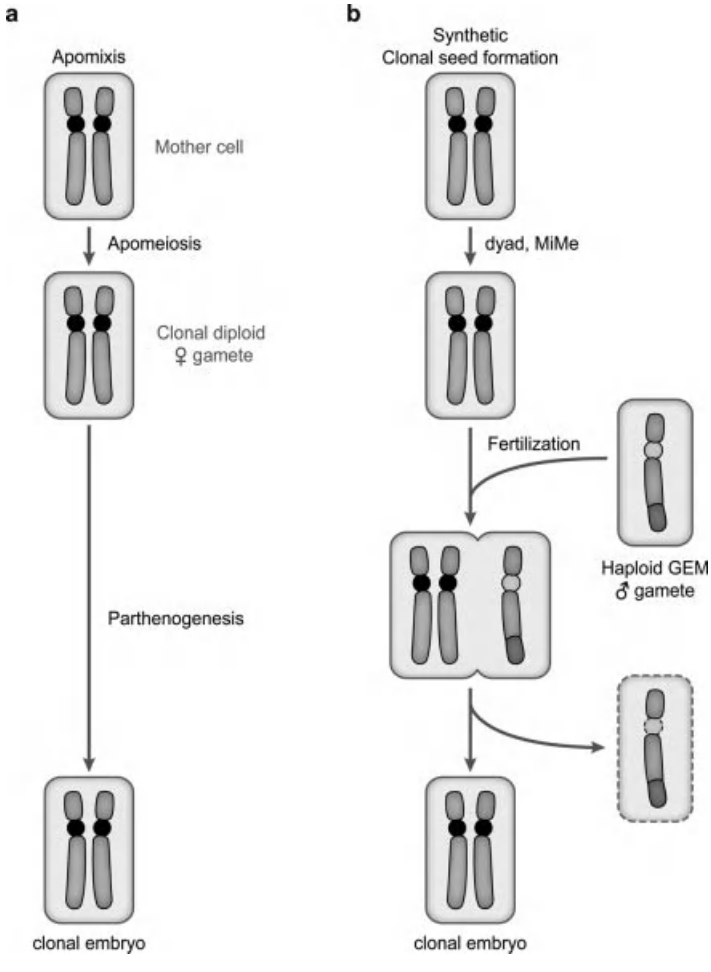

Figure 5.2 Natural and synthetic clonal reproduction through seed. *a,* In natural apomicts, clonal embryos with a maternal genotype develop without fertilization. *b,* Induction of clonal embryos in sexual plants as described by Marimuthu *et al.* (2011) involves combining mutants so that gametogenesis occurs without meiosis and subsequent fertilization with a parent whose chromosomes are modified to be eliminated after fertilization. (Modified from Marimuthu *et al.*, 2011.) (For color detail, see color plate section.)

engineer an equivalent for the outcome of apomixis by alterations in a small number of genes whose molecular identity is known.

Limitations of the system are that in some combinations the sexual pathway is no longer active in these plants as occurs in apomicts and is not easily reversible. Flexibility to switch between sexual and apomictic reproductive modes in a plant is a clear advantage for breeding apomictic crops containing multiple performance traits. The efficiency of clonal seed formation in this synthetic system is also lower than found in many apomicts, and it still relies on crossing, whereas to achieve the benefits of apomixis, the plants would need to propagate themselves without having to perform crosses. It may be possible to address some of these issues through further improvements in the methodology.

Conclusion and Future Prospects

It is apparent that the development of apomixis will require a biotechnological approach to install it in crops for which true-breeding hybrids provide a performance advantage. Analyses of apomixis control regions have shown that in several cases, loci controlling apomixis show complex structure and organization. In several species that exhibit apparently simple Mendelian inheritance of apomixis it is likely that apomixis control regions harbor more than one genetic determinant for the control of apomixis. Apomixis control in *Hieracium* and *Tripsacum* appears to be overlaid onto a background of sexual reproduction where loss of apomixis results in reversion to sexuality. These findings are consistent with the notion that apomixis results from altered control of the normal sexual program resulting in truncation or omission of events, or changes in timing of developmental events, rather than a substitution or mutational inactivation of sexual processes. The precise genetic determinants within apomixis control regions that are responsible for the trait remain to be identified. Partial sequencing of the DNA from these regions in *Pennisetum* and *Hieracium* shows the regions to be gene poor and abundant in repeat elements and transposons.

Parallel to the study of natural apomicts, analysis of sexually derived mutations has identified a number of genes whose mutation or altered expression results in phenotypes that resemble components of apomixis. Several of these genes are implicated in epigenetic control of gene expression by modification of DNA or chromatin and taken together point to the possibility that apomixis is under epigenetic control, although direct evidence in support of this remains to be obtained in the range of apomicts under study.

It has recently been possible to combine mutant alleles of molecularly characterized genes that result in apomixis related phenotypes in *Arabidopsis* to produce clonal seed, which demonstrates that it ought to be possible to synthesize apomixis in sexual plant species by manipulation of a small number of genes. Given the diversity of developmental mechanisms by which apomixis occurs, it is likely that there will be multiple ways in which apomixis may be developed as a technology for fixing heterosis and other desirable traits in crops to enable increased productivity under variable environmental conditions. Information on how to do this will come from studies on both natural apomicts and sexual model systems and strategies will also depend on agronomic features of the target crop.

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6 High-Throughput Genetic Dissection of Seed Dormancy

Jose M. Barrero, Colin Cavanagh, and Frank Gubler

Introduction

Seed Dormancy – Why It Is Important?

The success of flowering plants in most ecosystems could be seen as a result of the emergence of the seed structure as a reproduction vehicle. If plants are broadly defined as immobile living organisms, in contrast to animals, the seed would be the exception to that definition. The seed, enclosing an embryonic plant and nutrient sources, represents the final stage of the plant reproduction and allows the safe dispersion of the progeny. For doing that, the seed needs to survive a challenging and changing environment and to preserve the next generation until conditions are favorable for survival.

The delay between seed formation and seed germination is one of the most important times during the entire plant cycle and has to be carefully synchronized with the environment to maximize seedling survival. This timing is principally determined by seed dormancy, which is a biological condition (physiological, morphological, and physical) that temporarily blocks germination, keeping the seed quiescent (Baskin and Baskin, 2004). Physiological dormancy is the most common form and generally includes components of embryo- and seed coat–based dormancy. Seed dormancy is an adaptive trait with high variability across species that has enormous importance in both wild and domesticated plants. Seed dormancy programs determine the ecological niche in which the seed germinates and prospers and are related to different factors such as climate, moisture, soil characteristics, light, temperature, nutrients, abiotic and biotic stress factors, and many others (Finch-Savage and Leubner-Metzger, 2006). In domesticated species, the control of seed dormancy is crucial and influences important agricultural traits, such as uniform germination and stand establishment, preharvest sprouting susceptibility (Gubler *et al.*, 2005), and seed storage requirements.

Gene Regulation of Seed Dormancy

During their formation, seeds undergo dramatic changes in their developmental program, becoming dry latent structures from highly metabolic tissues. The genetic program regulating germination in seeds is influenced by the life history of the mother plant and external environment. Given the right

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environmental conditions, a nondormant seed germinates and grows, resulting in an independent autotrophic organism. During the transitions leading to germination, radical changes in hormone and protein content occur, along with changes in transcriptome profiles. Gene regulation is essential for dormancy control. There are several lines of evidence based on mutagenesis, global gene expression studies, and chemical inhibition that indicate that the messenger RNA (mRNA) that is stored late in seed development, before desiccation, will have a major role in determining the dormancy level of the seed (Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008a; Barrero *et al.*, 2010a; Weitbrecht *et al.*, 2011). Rajjou *et al.* (2004) found that seeds cannot complete germination in the presence of translational repressors, whereas *de novo* transcription was not required. Those results indicate that protein synthesis is required for germination, and consequently the stored mRNA population in the seed may have a key significance. This population of stored mRNA may be determined by the conditions in which the seed completed its development, and this influences the depth of seed dormancy. In relation to that, research in several species using mutants clearly shows that mechanisms controlling gene translation, especially those related to post-transcriptional RNA processing, maturation, and metabolism, are very important in the regulation of seed dormancy (Lu and Fedoroff, 2000; Hugouvieux *et al.*, 2001; Xiong *et al.*, 2001; Nishimura *et al.*, 2005; Grasser *et al.*, 2009; Narsai *et al.*, 2011). For example, the *hyl1* insertion mutation which knocks out miRNA processing, results in ABA hypersensitivity in seeds (Lu *et al.*, 2002).

Seed Dormancy Complexity Is a Scientific Challenge

General mechanisms or pathways associated with seed dormancy have been described, mainly in the model plant *Arabidopsis*. Microarrays have been performed to profile gene expression in dormant and nondormant seeds (from different ecotypes) along with comparison of dormant and after-ripened seeds from the same ecotype in *Arabidopsis* (Cadman *et al.*, 2006; Carrera *et al.*, 2007). However, a comprehensive model of the dormancy development program that explains how seeds acquire their dormancy, how it is maintained, and how it is broken down to allow germination has yet to be determined. The key genetic elements that keep a seed under the control of the dormancy program or the elements that are needed to initiate the germination program are unknown (Nonogaki *et al.*, 2010). Although dormancy has been intensively studied for a long time in the model plant *Arabidopsis* and in other species, we are only starting to understand what elements govern seed dormancy, and the manipulation of this trait is still a challenge. The complex web of genetic and environmental factors involved in seed dormancy and germination is wide, which could be a reason why reductionist approaches to analyzing seed dormancy have not provided a clear model explaining seed dormancy. The elements determining the dormancy level and the elements triggering germination are probably composite signals that result from interactions between genes and environment. New platforms and technologies for sequencing genomes and transcriptomes have the potential to dissect those complex interactions accurately and rapidly. Also, new computing power and visualization tools allow a more global analysis, combining systems biology approaches and coexpression studies for taking advantage of a full set of publicly available datasets. Seed dormancy needs to be studied in the context of the seed cycle because it is an intermediate step between seed maturation and germination (Gutierrez *et al.*, 2007; Angelovici *et al.*, 2010).

This chapter focuses on the genetic dissection of seed dormancy, more specifically on gene expression. We highlight how new high-throughput platforms and associated techniques can be used for revealing some of the complex mechanisms behind seed dormancy. Finally, we also

review strategies for gene discovery that can be used for mapping and identifying dormancy-related genes in crop species, where the genetic dissection and manipulation of seed dormancy are even more complex.

Profiling of Transcriptomic Changes

Global gene expression analyses using microarrays have been used for dissecting several aspects of seed dormancy. Several reviews summarize the findings of those approaches (Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008a, 2008b; Barrero *et al.*, 2010a). The novelty of these genome-wide approaches was their capability of identifying gene expression profiles or fingerprints associated with the dormant and the nondormant states. Specific gene expression profiles of dormant and nondormant (after-ripened) seeds were first described for the *Arabidopsis* Cvi ecotype (Cadman *et al.*, 2006). ABA-, seed maturation– and stress-related genes were abundant in the group of genes associated with dormancy. Genes related to protein metabolism, reserve mobilization, and cell wall modification were abundant in the after-ripened set. A similar transcriptomic scenario was described in the *Ler* ecotype (Carrera *et al.*, 2007), which produces seeds with weaker dormancy than Cvi, indicating that similar changes occur during after-ripening in different ecotypes, regardless of their level of dormancy. This work introduced an interesting new tool for analyzing and visualizing the gene datasets. Because the typical *Arabidopsis* gene ontology (GO) (Gaudet *et al.*, 2009) annotation provided limited understanding regarding which class of genes was important in dormancy and germination, the authors reannotated the genes in relation to previously described roles in germination- and dormancy-related terms (Microsoft Excel TAGGIT macro) (Carrera *et al.*, 2007). This TAGGIT workflow was used for reanalyzing new and previous microarray data and has been shown to give a distinct visual gene signature for dormant and after-ripened seeds (Holdsworth *et al.*, 2008b). Also, it was used for reanalyzing microarray data generated from dry and imbibed Col-0 seeds (Nakabayashi *et al.*, 2005) providing distinct gene signatures for both conditions. In relation to the gene expression changes found during early imbibition of Col-0 seeds, it was found that genes with differential expression between imbibed and dry seeds tended to be located in chromosome clusters, suggesting that epigenetic mechanisms could be involved in the regulation of those areas (Nakabayashi *et al.*, 2005).

Microarrays also have been used for identifying gene expression changes in response to environmental factors that alter dormancy (Finch-Savage *et al.*, 2007) including after-ripening, low temperature, nitrate, and light. All those factors promote germination in *Arabidopsis,* and the transcriptomic results indicated that with the exception of after-ripening, all the other factors have similar effects on transcriptomic profiles. The after-ripening mechanism has been investigated in several studies but remains poorly understood (Iglesias-Fernandez *et al.*, 2011). Part of the problem is the difficulty of separating the dormancy and germination programs from the after-ripening per se. Studies using mutants have demonstrated that the after-ripening is a genetically controlled process and that it occurs independently of germination and dormancy. Mutant seeds that cannot complete germination, such as *comatose-1* (*cts-1*), or that do not develop seed dormancy, such as *aba deficient 1-1* (*aba1-1*) or *aba insensitive 1-1* (*abi1-1*), undergo similar after-ripening changes as wild-type seed, showing similar gene expression signatures (Carrera *et al.*, 2007, 2008).

Microarray-based transcription studies have been used for spatial profiling of transcriptomes from different seed tissues. For example, gene expression changes have been profiled in the *Arabidopsis* endosperm and embryo during germination (Penfield *et al.*, 2006), indicating a very different response in those tissues. This finding is not surprising because it is clear that different tissues

of the seed have different contributions during seed dormancy and germination (Finch-Savage and Leubner-Metzger, 2006). In this sense, tissue-specific approaches can discover some genetic regulation that could be masked under the transcription of the whole seed. This tissue-specific microarray approach was performed in barley to compare dormant and after-ripened grains during early imbibition (Barrero *et al.*, 2009). Based on the results, it is proposed that the coleorhiza (a specific tissue in the embryo that surrounds the seminal roots) plays a major role in dormancy in cereals, acting as a physical barrier to root emergence in dormant grains. Similar to endosperm of dicotyledonous seeds, the coleorhiza cells undergo cell degradation in imbibed after-ripened seeds, allowing the root to emerge from the grain. Another more recent study used a similar approach in *Lepidium* for examining the role of the radicle and the micropylar endosperm cap during germination (Morris *et al.*, 2011). Both works indicate that genes involved in fundamental pathways controlling dormancy and germination, such as hormone metabolism and responsiveness or cell wall modification, are expressed in a very tight spatial and temporal pattern within the seed. These studies highlight the importance of accurate quantification of RNA populations in both time and space frames for understanding changes in seed dormancy. It is likely that translation and mRNA degradation may also be differentially regulated across different seed tissues and developmental stages.

These tissue-specific studies can now be performed with more accuracy and in a relatively high-throughput way using a new technique called INTACT (Isolation of Nuclei Tagged in Specific Cell Types) (Deal and Henikoff, 2010, 2011). This technique consists of tagging the nuclear envelop of specific cell types. This tag is a biotinylated protein, and the nuclei of interest can be isolated on extraction with the use of streptavidin-coated magnetic beads. This straightforward technique does not require expensive equipment and results in high purity of isolated nuclei. Although it has been applied only in *Arabidopsis*, it can potentially be used in any other plant species and could allow the dissection of seed dormancy at the cellular level.

Apart from microarrays, other high-throughput platforms are available to study the expression of all the transcription factors from *Arabidopsis* and rice by real-time reverse transcriptase polymerase chain reaction. This platform has been used in *Arabidopsis* for analyzing the dormant and nondormant states using C24 seeds (Barrero *et al.*, 2010b). This strategy allows the study of dormancy-related transcription factors that are often undetectable in microarray approaches because of their low expression values.

Use of New Sequencing Platforms and Associated Techniques to Study Seed Dormancy

The new high-throughput platforms for sequencing nucleic acids (known as next generation sequencing) (Lister *et al.*, 2009) are having a large impact in many fields of plant molecular biology, including seed dormancy (Weitbrecht *et al.*, 2011). Although there are not yet many studies using next generation platforms to analyze seed dormancy, they may have the potential to unmask the key elements controlling the dormancy program and dissecting poorly understood processes such as dry after-ripening or the induction of secondary dormancy.

The detailed analysis of the mRNA populations stored in the seed and how these populations change depending on the maturation conditions, during after-ripening or in response to imbibition, is now readily achievable using next generation sequencing. Of special interest is the capability of the next generation platforms for studying mRNA decay. Gene expression is a result of the balance between mRNA transcription and decay. Although the first has been extensively studied in seeds, the second has been mainly ignored, even when mutant approaches have provided strong support for

the importance of mRNA stability in dormancy and germination. For example, *Arabidopsis* mutants affecting RNA capping (Hugouvieux *et al.*, 2001), mRNA splicing and degradation (Xiong *et al.*, 2001), and mRNA polyadenylated tail stability (Nishimura *et al.*, 2005) all have altered dormancy. The lack of study of mRNA decay has been partially due to difficulties in detecting it without the confounding impacts of transcription on transcript abundance. More recent studies have highlighted the importance of different mRNA decay pathways during plant growth and development, and the degree of specificity that different decay pathways show for regulating different biological processes during plant development (Belostotsky and Sieburth, 2009). General mRNA decay mechanisms include the 5′ decapping followed by a 5′-to-3′ exoribonuclease, the 3′ deadenylation followed by the exosome 3′-to-5′ digestion, and the internal cleavage followed by degradation in both directions. Prior analyses of these processes on a genome-wide scale discovered interesting details about how the life span of different mRNA species varies and how some sequences and motifs may be more susceptible to increased or reduced decay (Narsai *et al.*, 2007). Microarray studies were used to analyze the degradation of uncapped mRNA (Jiao *et al.*, 2008), but this technique had limitations for unmasking the contributions of specific decay pathways and for a reliable quantification of the mRNA population. Microarray detection and quantification is dependent on the number of probes on the microarray chip and their positions along the transcript. The new deep sequencing approaches can generate a continuous series of overlapping reads along a transcript, and the analysis of distorted distributions of those reads along transcript length could be a signature for 5′ or 3′ decay processes. Another important advance is that the absolute number of reads can be easily normalized against an external control RNA molecule, providing an opportunity to monitor relative or absolute changes in mRNA abundance with accuracy.

Post-transcriptional regulation of gene expression by microRNA-directed degradation pathways is important in many stages of plant growth and development. So far, only two microRNAs (miRNA 159 and 160) have been shown to be involved in the regulation of seed dormancy and germination (Liu *et al.*, 2007; Reyes and Chua, 2007). Current sequencing systems have the capability to reveal the degradome characteristics of mRNA populations (Meng *et al.*, 2010). Using the 5′-rapid amplification of cDNA ends (RACE), cDNA libraries of the 5′ ends of polyadenylated messengers can be readily constructed and sequenced providing a full map of the microRNA-targeted degradome. This method, called parallel analysis of RNA ends (PARE) (German *et al.*, 2008), has become popular for validating known microRNA targets and for finding new ones. This approach has been used for analyzing the microRNAs involved during seed development and germination in rice and maize (Xue *et al.*, 2009; Wang *et al.*, 2011), and it could be applied for analyzing several aspects of seed dormancy.

In combination with polysome or ribosome profiling, the new sequencing platforms allow for the identification of mRNAs that are associated with ribosomes (Ingolia *et al.*, 2009). This would generate an indirect estimation of the translation happening in a given tissue at a given time, providing a snapshot of the number of 30 nucleotide footprints occupied by ribosomes per mRNA species. mRNAs that are being actively translated have a higher number of ribosomes associated with the mRNA than those that are not being translated. The use of this technique would allow the identification of the population of mRNAs that is stored in the seed associated with ribosomes or which mRNAs are potentially translated in imbibed dormant or after-ripened seeds.

To be effective, next generation sequencing approaches need to be standardized across the seed biology community. Library construction and replication should be unified to compare results. In addition, experimental conditions used in dormancy experiments need to be standardized to allow datasets to be combined for future analyses. Protocols for different seed dormancy studies have been published more recently (Kermode, 2011).

Visualization Tools

The capability to analyze and visualize genome-wide results is limited. Microarray datasets composed of thousands of data points are difficult to visualize and summarize as a whole set, and often only partial analysis of a particular group of genes is given, leaving a large proportion of the data not fully analyzed. Next generation experiments are increasing that problem as the data points coming from the new platforms are counted in millions. Only with the use of computing power and new visualization tools will it be possible to achieve a global analysis of the datasets.

The visualization of the molecular processes associated with seed dormancy would facilitate the complex and comprehensive analysis of changes happening under different conditions. Common tools used to visualize transcriptomic changes, such as the MapMan and PageMan packages (http://MapMan.gabipd.org), have been implemented with new pathways related to seed dormancy and germination (Joosen *et al.*, 2011). These tools are expected to allow a more complete and accurate visualization and analysis of array datasets.

Of special interest is the Bio-Array Resource (BAR) developed by the University of Toronto (http://bar.utoronto.ca/welcome.htm). The BAR contains a series of Web-based tools for visualizing gene expression and protein changes. The electronic fluorescent pictographic (eFP) browser (Winter *et al.*, 2007; Bassel *et al.*, 2008) has proven to be of great value for seed scientists. The BAR website contains microarray information from dozens of seed germination experiments under different conditions and dormancy release treatments. It also allows the clustering of a gene of interest with the genes included in several TAGGIT ontology categories (Carrera *et al.*, 2007). Several other eFP browsers are also available for other species, such as poplar, *Medicago*, rice, barley, maize, and soybean.

Coexpression Studies and Systems Biology Approaches

In addition to single gene expression analyses, visualization tools have been developed to explore the interactions of cohorts of genes that share distinct expression profiles. This approach for studying gene coexpression could be very important for finding new genes involved in the control of seed dormancy and even more so in species where the gene annotation is poor. The Virtual Seed Web Resource (http://vseed.nottingham.ac.uk) contains tools for the analysis of gene networks during seed maturation, dormancy, and germination. The SeedNet network model (Bassel *et al.*, 2011) uses publicly available seed expression data from many *Arabidopsis* samples to build a genome-wide gene association clustering. This is a powerful tool that takes advantage of experiments performed by the scientific community to generate hierarchical associations between genes on the basis of their expressions in multiple seed-related analyses. SeedNet can accurately find novel seed germination and dormancy regulators and predict genetic interactions between them. In the future, the website will incorporate similar modules for the dissection of genetic interactions in a tissue-specific manner in the micropylar endosperm of *Arabidopsis* (MENet) and in the seminal root apical meristem (AxisNet). Conserved networks will also be identified by comparing gene datasets from wheat grain development and in response to abiotic stress (WheatNet). The Virtual Seed Web Resource is also implementing eFP browsers for the visualization of high-resolution gene expression changes in different tissues of germinating *Arabidopsis* and *Lepidium* seeds (Linkies *et al.*, 2009; Morris *et al.*, 2011).

Finally, a systems biology perspective can be used to formulate different scenarios related to seed dormancy and to predict the outcome of complex gene expression. This approach has been used to explore the balance and crosstalk between gibberellins and ABA, taking into account different

environmental conditions (Penfield and King, 2009). It allows the formulation of different models that can be experimentally tested.

Mapping Populations for Gene Discovery

Marker-Assisted Gene Discovery

The study of seed dormancy in crop species such as cereals and the modification of this trait by breeding strategies has lagged behind the rapid progress made in model plants such as *Arabidopsis*. The use of molecular markers for improving cereal breeding has had the most success in tagging loci for large effect or Mendelian traits, such as disease resistance through the selection of markers in early-generation breeding material (e.g., F2 or early backcrosses). A far more difficult endeavor is the implementation of marker systems for polygenic traits (Baenziger *et al.*, 2006; Bernardo, 2008), such as seed dormancy. Typically, major QTLs, such as the QTL in the chromosome 4AL in wheat (Mares *et al.*, 2005), have been implemented in breeding, but often several QTLs need to be combined to be effective. The challenge for gene cloning, using traditional biparental mapping populations, either recombinant inbred lines (RILs) or doubled haploids, is that the support intervals for the QTL are typically very large. This makes it difficult to identify candidate genes or to implement them in marker assisted selection. To ensure with confidence that a given QTL is being selected, large regions of the genome are selected, and the sections of the genome are unable to recombine. Genetic background also affects the strength of a given QTL (Jannink *et al.*, 2009). There are dozens of QTL studies for seed dormancy in many species from *Arabidopsis* to wheat (Anderson *et al.*, 1993; Groos *et al.*, 2002; Alonso-Blanco *et al.*, 2003; Mares *et al.*, 2005). So far in *Arabidopsis*, only one candidate QTL gene, *Delay of Germination 1* (*DOG1*) (Bentsink *et al.*, 2006), has been cloned, and the protein has an unknown function. In rice and wheat, genes responsible for major QTLs involved in grain dormancy and testa color has been also identified (Gu *et al.*, 2011; Himi *et al.*, 2011). In wheat *Tamyb10* and in rice *Rc*, a basic helix-loop-helix transcription factor acts on the same flavonoid biosynthetic pathway responsible for the red testa color. Several other highly significant dormancy QTLs have been known for some time, and their molecular identification remains to be elucidated. Interspecies approaches for comparing syntenic regions are expected to become more common because the new sequencing platforms will provide more genomic sequences, and this will facilitate the cloning of QTLs (Somyong *et al.*, 2011).

Genome-wide Association Studies

Genome-wide association (GWAS) mapping has been used more recently as a method for detecting marker-trait associations (Mackay and Powell, 2007). Success using this approach has been mixed depending on many factors, including the availability of a sequence, size of the genome, polyploidy, and availability of high-throughput markers. In species where a genome sequence is available (e.g., *Arabidopsis* and rice), greater success has been achieved (Atwell *et al.*, 2010; Huang *et al.*, 2010).

The major advantage of GWAS is that it may be implemented within a breeding program or existing varieties, making it a very attractive option for cereal breeders. Numerous public initiatives are currently underway in wheat (http://www.triticeaegenome.eu/) and barley (http://www.agoueb.org/) (Waugh *et al.*, 2010). GWAS also has the advantage that the resolution is much higher than linkage analysis in biparental populations owing to the many generations of recombination captured in the population. This resolution generally means that an association is detected only when the marker

is in tight linkage to the QTL; this increases the need for greater marker density. However, with the new sequencing capabilities available, high density marker coverage can be achieved using low pass resequencing (Huang *et al.*, 2010).

One disadvantage of GWAS is the need to control for population structure, which causes an increase in the number of false-positives (Cockram *et al.*, 2010). This problem can be partially alleviated through appropriate statistical modeling (Yu *et al.*, 2006). Association mapping can suffer from the factors that generate its precision, and for greater resolution a greater population size is required to exploit the historical recombination. Although population structure may be accounted for within a model, the risk of not identifying an important QTL that is associated with population structure will also occur.

Alternative Mapping Approaches

More recently, alternative approaches to marker-trait association identification have been proposed in experimental populations. These populations differ from the traditional approaches in that they include multiple founders and are large in size. The first is the nested association mapping (NAM) (Yu *et al.*, 2008) approach, where a series of lines are crossed to a common founder. In maize, NAM has been done using a common parent and 25 lines that were crossed generating 200 RILs per cross for a total of 5000 RILs in the population. NAM uses the power of linkage analysis (within cross) with the precision of association mapping (between families). Results are now emerging (Elshire *et al.*, 2011, Tian *et al.*, 2011), and the ability to impute marker information from a relatively sparse genotyping effort is possible in populations where a genome sequence is unavailable. Such an approach allows many founders to be used and for a trait such as dormancy allows the incorporation of a wide array of germplasm known to contain varying levels of dormancy.

The second approach being implemented is the multiparent advanced generation inter-cross (MAGIC) (Cavanagh *et al.*, 2008). This approach has been implemented in many plant species such as *Arabidopsis* (Kover *et al.*, 2009; Huang *et al.*, 2011), rice (http://irri.org/news-events/irri-news/irri-magic-team-unveils-genetic-diversity), and wheat (http://www.csiro.au/science/MAGIC), and results are beginning to emerge regarding the power of this approach for gene-trait associations. MAGIC uses multiple founders crossed in a balanced fashion to create a set of progeny lines, each having a mosaic of the founder genomes. This approach has already shown success in the identification of genomic regions controlling, among other traits, days to germination with a high degree of accuracy based on previously known genes (Kover *et al.*, 2009). MAGIC provides the ability to look at additive gene effects simultaneously along with epistatic effects. A major difference with other population designs is that each allelic effect can be assessed in a range of genetic backgrounds that occur within the population, without the need to worry about the effects of population structure. In addition, a series of near isogenic lines may be generated immediately from the RIL population after QTL identification. By identifying heterozygous individuals (for a specific QTL) within the population, the effects resulting from a range of genetic backgrounds may be tested. This is a very powerful feature of MAGIC, allowing a trackable approach to understanding the genetic factors underlying complex traits, without the need to generate further genetic resources.

Perspective

The impact of next generation sequencing is expected to speed up the identification of novel genes and alleles that control seed dormancy. Those platforms have the capability to dissect complex

processes related to seed dormancy, such as after-ripening, which is going to be possible on a variety of species and conditions. Already the power of these technologies is speeding up the fine mapping of candidate QTL genes in nonsequenced crops. This will lead to widespread introgression of high dormancy germplasm, which will deliver crops that are resistant to preharvest sprouting. It is expected that cutting-edge technologies, such as cell-specific approaches, will help define the contribution of various cell types to the overall seed dormancy and help to unmask its control mechanisms. Future studies are likely to focus on how these various cell types interact to determine whether a seed germinates or remains dormant.

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7 Genomic Specification of Starch Biosynthesis in Maize Endosperm

Tracie A. Hennen-Bierwagen and Alan M. Myers

Introduction

Plant reproductive function in many species relies on storage of glucosyl units in the seed in the form of starch granules. This storage is particularly relevant in cereal crops, in which endosperm tissue forms most of the seed mass. Human use of cereals for food and renewable raw materials has provided strong selection for higher yields, which are generated in large part by increased endosperm mass and elevated starch content within that tissue. As a result, endosperm metabolism in cereal crops has evolved to provide an extremely high sink strength for forming starch from photosynthate generated in the leaves and transported to the seed as sucrose. Metabolic flux into starch is stimulated by the crystallization of glucan polymers into starch granules, removing the product of the pathway from the reaction equilibrium. In addition to seed storage tissues, starch granules accumulate in photosynthetic tissues where they are formed and then degraded regularly throughout the diurnal cycle. Seed starch metabolism uses the same biosynthetic pathway and often the same enzymes as those employed in leaves; however, granule formation and growth continue over the course of development leading to the vast glucose stores in mature endosperm. Thus, an important function of plant genomes is to encode the starch metabolic pathway that operates in the seed.

Genetic research into starch biosynthesis began in essence with Mendel's identification of the pea *rugosus* (*r*) locus, which encodes a starch branching enzyme (SBE) (Bhattacharyya *et al.*, 1990). Maize genes encoding components of the starch biosynthetic pathway were identified shortly thereafter from scorable changes in seed phenotype conditioned by mutant alleles (e.g., *sugary1* [*su1*] and *waxy* [*wx1*], identified in 1901 and 1909) (Correns, 1901; Collins, 1909). Ensuing forward genetics research identified loci encoding enzymes that function in each step of the starch biosynthesis pathway. Enzyme purification coupled with molecular cloning studies revealed the amino acid sequences of numerous starch biosynthetic enzymes. Using this information to search the maize genome sequence revealed additional starch biosynthetic enzymes that had not been identified by mutations, and reverse genetics yielded loss-of-function alleles in starch biosynthetic genes that had not been found by classical genetics. From this body of research, a complete characterization of essentially all of the genetic functions that constitute the endosperm starch biosynthetic pathway is now available.

Starch is made up of deceptively complex molecules. The polymers that constitute starch are composed of only glucose subunits linked together with either $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow6)$ glycoside bonds. However, the precise arrangement of these bonds, exhibited in the linear chain length distribution

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and the locations of branch points, can have dramatic effects on physical properties such as gelling temperature or digestability. These properties affect the applicability of different starches to various end uses and have economic significance. A combination of biochemical, genetic, and genomic studies is beginning to reveal the metabolic mechanisms that regulate these important properties.

This chapter briefly describes the molecular structure of starch and the enzymes that assemble glucose units into the polymers contained within starch granules; the reader is referred to in-depth reviews for a full treatment of the subjects. The chapter then presents a survey of the maize genome to describe each genetic element that encodes an enzyme involved in starch biosynthesis. The genes that are responsible for starch biosynthesis particularly in endosperm tissue are highlighted, and the likely evolutionary history leading to the genomic specification of starch biosynthesis in maize is described.

Overview of Starch Biosynthetic Pathway

Starch Structure

For reviews of starch structure, the reader is referred to Buléon *et al.* (1998), Zeeman *et al.* (2010), Tetlow (2011), and references therein. Starch accumulates in insoluble granules composed of homopolymers in which glucosyl units are linked predominantly by $\alpha(1\rightarrow4)$ glycoside bonds into chains. Such "linear" chains can be joined to each other by $\alpha(1\rightarrow6)$ glycoside bonds, introducing "branches" into the polymer structure. Two types of glucan polymer are present in starch granules. Amylose is almost entirely linear, with only a very slight frequency of branch linkages relative to the number of $\alpha(1\rightarrow4)$ bonds. Amylopectin, in contrast, is branched at a frequency of approximately 5% and displays a bimodal distribution of linear chain lengths. The relative abundance of amylose and amylopectin varies by species and tissue. In cereal endosperm starch, the granules are made up of ~75% amylopectin and 25% amylose. The structure of amylopectin imparts the semicrystalline nature of starch granules because mutants that lack amylose produce particles similar in size and shape to particles found in wild-type plants. Within amylopectin, the branch linkages are thought to be clustered near each other so that adjacent linear chains lacking branches are able to associate with each other and crystallize owing to hydrogen bonding and hydrophobic effects. Amylopectin is "semicrystalline," meaning that crystalline regions alternate with amorphous regions in which the branch linkage clusters are located. The primary crystalline units assemble into several levels of higher order structures, eventually leading to insoluble starch granules.

The enzymes that catalyze starch synthesis must be able to function with structural specificity. Branch linkages are not randomly located but rather are situated relatively close to each other, leaving unbranched regions in other areas of the polymer. The frequency of branch linkages is conserved among starches from different sources. Linear chain lengths are also specific within certain ranges, with some chains forming single crystalline units and others extending between crystalline layers to give rise to the bimodal distribution of chain lengths. Amylopectin structure can be compared with glycogen, which serves a glucose storage function in organisms other than plants. In glycogen, the chain length distribution is unimodal, the branch linkages are thought to be dispersed evenly throughout the polymer, and the branch frequency is ~10% – twice that of amylopectin. As a result of these structural differences, amylopectin adopts an insoluble semicrystalline structure, whereas glycogen is soluble within the cytosol. The fundamental structure of plant starches is the alternating regions of amorphous and crystalline lamellae, and this is conserved as a 9- to 10-nm repeat structure throughout the plant kingdom (Jenkins *et al.*, 1993). Owing to this conserved molecular architecture,

starch granules can store vastly more glucose units than glycogen and provide an essential element of seed physiology and plant reproduction.

Starch Biosynthetic Enzymes

For recent reviews addressing the mechanisms of starch granule biosynthesis, the reader is referred to Zeeman *et al.* (2010), Tetlow (2011), Jeon *et al.* (2010), Keeling and Myers (2010), and Hennen-Bierwagen *et al.* (2012). The metabolic pathways for conversion of sucrose to starch are summarized in Figure 7.1. Glucose-1-P (Glc-1-P) is generated either directly from sucrose through two enzymatic steps or indirectly by conversion of fructose into triose phosphates and then back into Glc-1-P. The committed step for starch biosynthesis is catalyzed by ADP glucose pyrophosphorylase (AGPase; ATP:α-D-glucose-1-phosphate adenylyltransferase), which converts Glc-1-P plus ATP into ADP glucose (ADPGlc) plus inorganic pyrophosphate (PPi). ADPGlc serves as the glucosyl unit donor to growing linear glucan chains. Chain elongation is catalyzed by starch synthase (SS; ADPGlc:(1→4)-α-D-glucan 4-α-D-glucosyltransferase), which transfers the glucosyl unit from ADPGlc to the nonreducing end of the glucose polymer and creates a new α(1→4) linkage. Branch linkages are introduced by starch branching enzyme (SBE; α(1→4)-glucan:α(1→4)-glucan-6-glycosyltransferase). SBE catalyzes cleavage of an α(1→4)-linkage in a linear chain and

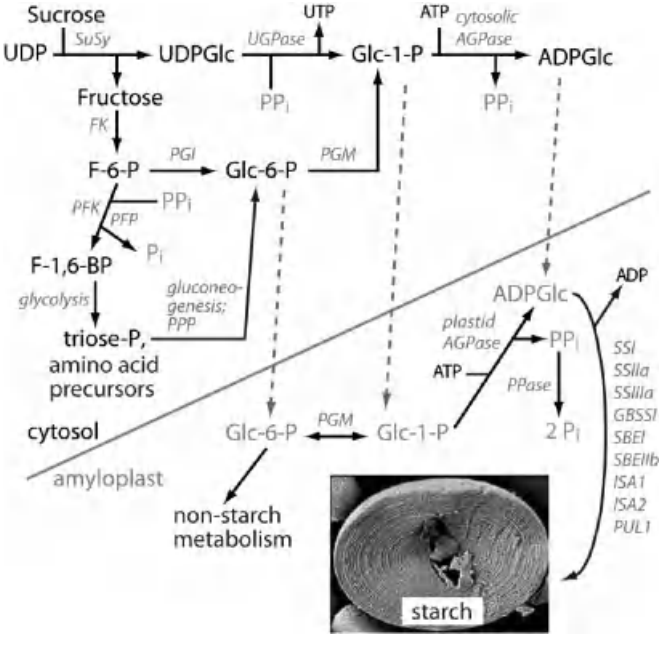

Figure 7.1 Outline of endosperm metabolism from sucrose to starch. *Gray lines* indicate transport from the cytosol into the amyloplast stroma. Glc-1-P is supplied either by UGPase in the "direct" path from sucrose or from metabolic conversion to triose phosphates and resynthesis back into hexose phosphate. Enzymes noted as being involved in starch biosynthesis have been identified by genetic analyses; this does not preclude the involvement of additional isoforms. SuSy, sucrose synthase; UGPase, UDP glucose pyrophosphorylase; FK, fructokinase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; PFK, phosphofructokinase; PFP, pyrophosphate-dependent phosphofructokinase; PPP, pentose phosphate pathway; PPase, pyrophosphatase; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-bisphosphate. (For color detail, see color plate section.)

reattachment of the reducing end of the released fragment to a 6-hydroxyl group of either the cleaved chain or a neighboring chain, introducing an α(1→6) bond. Starch debranching enzyme (DBE; α(1→6)-glucan hydrolase) also participates in starch biosynthesis, presumably by removal of a subset of branches introduced by SBE, forming the branch linkage arrangement thought to facilitate crystallization.

The concerted actions of SS, SBE, and DBE are responsible for generating the specific molecular architecture of amylopectin that enable crystallization and subsequent formation of the starch granule. However, the precise molecular mechanisms responsible for starch biosynthesis are unclear. A simple linear model would suggest that AGPase generates the glucose donor, SS produces linear chains, SBE creates branches, DBE removes selected branches, and finally crystallization occurs. However, such a mechanism does not seem likely to explain the specific placement of branch linkages or linear chain length distributions. Considering SS, SBE, and DBE, the products of any one reaction serve as the substrate for the other two reactions. A more realistic model suggests that SS, SBE, and DBE act simultaneously on a precursor polymer that crystallizes spontaneously and is removed from the reaction equilibrium. Crystallization and polymer growth may occur in different locations of a single precursor molecule. In support of this model, physical interactions among SS, SBE, and AGPase have been demonstrated (Tetlow *et al.*, 2004, 2008; Hennen-Bierwagen *et al.*, 2008, 2009).

Genomic Specification of Endosperm Starch Biosynthesis in Maize

The maize genome contains multiple elements encoding each of the enzymes involved in starch biosynthesis. These genes had their origin in the monophyletic event that incorporated a cyanobacterial endosymbiont to give rise to the plastid-containing organisms known as *Archaeplastida* (Deschamps *et al.*, 2008). Subsequent evolution led to divergence of the *Chloroplastida* species (i.e., green algae and land plants), distinguished by the presence of chloroplasts. At least one ortholog of each maize starch biosynthetic gene is conserved in all known *Chloroplastida* species, which indicates functional selection rather than gene duplication within specific lineages (Deschamps *et al.*, 2008). This analysis identifies conserved "classes" of each enzyme. In addition, in many instances, multiple paralogs (i.e., "isoforms") within a given class have arisen through gene duplication events. In some instances, duplicate genes (referred to as "twins") also exist within isoform groups in maize, as the result of a relatively recent whole-genome duplication specific to that evolutionary lineage. Table 7.1 specifies all known elements in the maize genome that potentially encode starch biosynthetic enzymes, each of which is discussed in the following sections.

AGPase

AGPase physiology in endosperm of grass species in the Poaceae family, including cereal crops, differs from that of other plant species and tissues owing to distinct subcellular locations of the enzyme (Geigenberger, 2011 and references therein). AGPase appears to be strictly plastidial in most plant tissues (Beckles *et al.*, 2001), whereas in cereal endosperms most AGPase activity is cytosolic and a minor isoform resides within the amyloplast (Rosti *et al.*, 2006 and references therein). Such an arrangement necessitates distinct modes of regulation and metabolic control of starch biosynthesis, including transport of ADPGlc by the protein encoded by the *brittle1 (bt1)* gene and transport of glucose phosphate (Glc-1-P and/or Glc-6-P) from the cytosol into the amyloplast (Figure 7.1).

Table 7.1 Known Elements in the Maize Genome That Potentially Encode Starch Biosynthetic Enzymes

Gene Name	Encoded Protein	Chromosome/ Coordinates[a]	Sequence References	Mutation References
sh2	AGPase LS	3R; 216,414,685–216,421,042	Bhave *et al.*, 1990; Shaw and Hannah, 1992	Mains, 1949
embL	AGPase LS	6F; 166,587,013–166,592,608	Giroux *et al.*, 1995	
leafL	AGPase LS	1R; 273,447,466–273,452,205	Yan *et al.*, 2009b; Huang *et al.*, 2010	Huang *et al.*, submitted
AGPL3	AGPase LS	7R; 23,756,240–23,767,010	Yan *et al.*, 2009b	
bt2	AGPase SS	4R; 58,954,361–58,961,481	Hannah *et al.*, 2001	Teas and Teas, 1953
embS	AGPase SS	2R; 173,380,830–173,391,388	Hannah *et al.*, 2001	Huang *et al.*, submitted
leafS	AGPase SS	1F; 221,648,603–221,653,116	Prioul *et al.*, 1994; Hannah *et al.*, 2001	Slewinski *et al.*, 2008
bt1	ADPGlc transporter	5R 112,928,080–112,930,204	Sullivan *et al.*, 1991	Mangelsdorf, 1926; Wentz, 1926
ss1	SSI	9F; 17,633,700–17,644,420	Knight *et al.*, 1998	
su2	SSIIa	6F; 113,234,637–113,238,773	Harn *et al.*, 1998	Eyster, 1934
ss2b-1	SSIIb-1	Not aligned[b]	Harn *et al.*, 1998	
ss2b-2	SSIIb-2	5R; 206,850,639–206,855,628	Yan *et al.*, 2009b	
ss2c	SSIIc	5R; 33,050,572–33,066,185	Yan *et al.*, 2009a	
du1	SSIIIa	10F; 59,506,710–59,518,288	Gao *et al.*, 1998; Lin *et al.*, 2012	Mangelsdorf, 1947
ss3b-1	SSIIIb-1	10F; 142,920,563–142,929,523	Yan *et al.*, 2009b	
ss3b-2	SSIIIb-2	2F; 8,871,415–8,890,970	Yan *et al.*, 2009b	
ss4	SSIV	8F; 125,308,442–125,317,219	Yan *et al.*, 2009b	
wx1	GBSSI	9R; 23,256,335–23,260,210	Shure *et al.*, 1983; Klösgen *et al.*, 1986	Collins, 1909
gbs2	GBSSII	7R; 34,872,733–34,879,973	Yan *et al.*, 2009b	
sbe1	SBEI	5R; 63,318,794–63,324,615	Baba *et al.*, 1991; Fisher *et al.*, 1995; Kim *et al.*, 1998	Blauth *et al.*, 2002
sbe2a	SBEIIa	2F; 58,586,007–58,596,220	Gao *et al.*, 1997	Blauth *et al.*, 2001
ae	SBEIIb	5R; 168,451,701–168,468,750	Fisher *et al.*, 1993; Stinard *et al.*, 1993	Vinyard and Bear, 1952
sbe3	SBEIII	8F; 141,990,059–141,993,024	Yan *et al.*, 2009b	
su1	ISA1	4F; 41,369,510–41,378,086	James *et al.*, 1995; Beatty *et al.*, 1997	Correns, 1901
isa2	ISA2	6F; 144,564,725–144,567,105	Dinges *et al.*, 2003	Kubo *et al.*, 2010
isa3	ISA3	7R; 129,101,533–129,113,095	Dinges *et al.*, 2003	
zpu1	PUL	2R; 108,631,513–108,681,578	Beatty *et al.*, 1999	Dinges *et al.*, 2003

[a]Chromosome coordinates refer to maize genome assembly RefGen_v2, December, 18, 2010 (http://www.plantgdb.org/ZmGDB/). The transcribed region is indicated.

[b]The cDNA encoding SSIIb-1 is specified by Genbank accession number AF019297 and is supported by multiple EST sequences in Genbank with exact sequence matches. However, the cDNA sequence does not align anywhere in the B73 maize genome sequence with near-perfect identity.

The genetic specification of AGPase in cereal species is complex and not fully understood. Plant AGPase is an $\alpha_2\beta_2$ heterotetramer of approximately 210 kDa, comprising two structurally related polypeptides referred to as the large subunit (AGPase LS) and small subunit (AGPase SS) (Morell *et al.*, 1987; Lin *et al.*, 1988; Okita *et al.*, 1990; Preiss *et al.*, 1990). The maize genome contains four genes encoding AGPase LS and three others that code for AGPase SS (Table 7.1). This set of genes has been described by multiple laboratories using various nomenclature systems. Table 7.1

defines each gene by its position in the maize genome and its nucleotide sequence, and the particular nomenclature used in this chapter is noted.

Mutations of the *shrunken2* gene (*sh2*), encoding AGPase LS, or *bt2*, encoding AGPase SS, cause reduction in endosperm starch content to ~25%–38% of normal (Laughnan, 1953; Cameron and Teas, 1954; Tsai and Nelson, 1966) and reduce total AGPase activity to ~15% of the wild-type value (Dickinson and Preiss, 1969). SH2 and BT2 are inferred to be cytosolic proteins from the findings that (1) when the BT1 ADPGlc transporter in the amyloplast membrane is defective owing to mutation, ADPGlc accumulates to high levels in endosperm cytosol; (2) cytosolic ADPGlc accumulation does not occur if *sh2* is also mutated in addition to *bt1*; and (3) neither BT2 nor SH2 possesses plastid transit peptides (Hannah, 2007).

The 15% of AGPase activity that remains in the absence of the cytosolic form could be encoded by a combination of any of the three other AGPase LS genes and two other AGPase SS genes present in maize (Table 7.1). Mutations of *embS* and *leafL* were obtained by reverse genetics and tested for effects on endosperm starch content (authors' unpublished results). In both instances, the starch content was reduced by ~6% compared with wild-type, indicating that the AGPase SS and AGPase LS encoded by these two genes contribute to AGPase activity within amyloplasts. Further analyses are necessary to test the possibility that *leafS*, *embL*, and *AGPL3* also provide plastid AGPase function in the endosperm. The dual localization of AGPase is conserved in cereal species and is presumed to provide a selective advantage. A possible explanation for such a role is modulation of the sink strength of endosperm tissue so that hexose phosphate present in the plastid can be used either for starch biosynthesis or for nonstarch metabolism (Figure 7.1).

Estimates of the relative steady-state mRNA levels determined from RNA-seq data (www.maizegdb.org) can provide evidence regarding which genes code for the AGPase activity in specific tissues. *SH2* and *BT2* transcripts are highly expressed in endosperm, with only trace amounts in embryo and essentially no transcript present in any other tissue. These data support the conclusion that cytosolic AGPase is specific to endosperm physiology. Transcripts from all five of the other genes encoding AGPase subunits accumulate to varying degrees in endosperm, although AGPL3 mRNA abundance is far lower than that for all of the other genes. These data are consistent with potential functions of all the maize AGPase genes in contributing to amyloplast AGPase activity. Multiple AGPase genes function in embryo and leaf, as shown by the fact that *embS* or *leafL* mutations reduce but do not eliminate starch content in those tissues (authors' unpublished results).

Evolution of the AGPase genes in maize has been investigated by phylogenetic comparisons (Figure 7.2*A*) (Rosti and Denyer, 2007; Georgelis *et al.*, 2008; Yan *et al.*, 2009b). AGPase LS and AGPase SS proteins from *Chloroplastida* species (i.e., green algae and land plants) are all >50% identical to each other at the amino acid level over the entire catalytic domain. These genes arose from a common ancestor present in a progenitor of the extant chloroplast-containing organisms. Duplications gave rise to at least three genes encoding AGPase LS before divergence of monocots and eudicots, as shown by orthologs present in maize, rice, *Arabidopsis*, and poplar. The maize genes *bt2* and *embS* are 90% identical, and each has an ortholog in the rice genome, which suggests that they arose as the result of a whole-genome duplication early in the evolution of the Graminacea. Subsequently, there was a second whole-genome duplication specific to maize, and this gave rise to the twin genes *bt2* and *leafS*. Accordingly, *leafS* is not present in rice. Among the four maize genes encoding AGPase LS, *sh2* and *embL* are more closely related to each other than to *leafL* or *APGL3* and are both present in rice. Thus, *sh2* and *embL* are thought to be derived from whole-genome duplication in an ancestral grass species. Orthologs of *leafL* and *AGPL3* are present in rice, maize, and eudicot species, indicating their presence in a primordial angiosperm before divergence of the monocots.

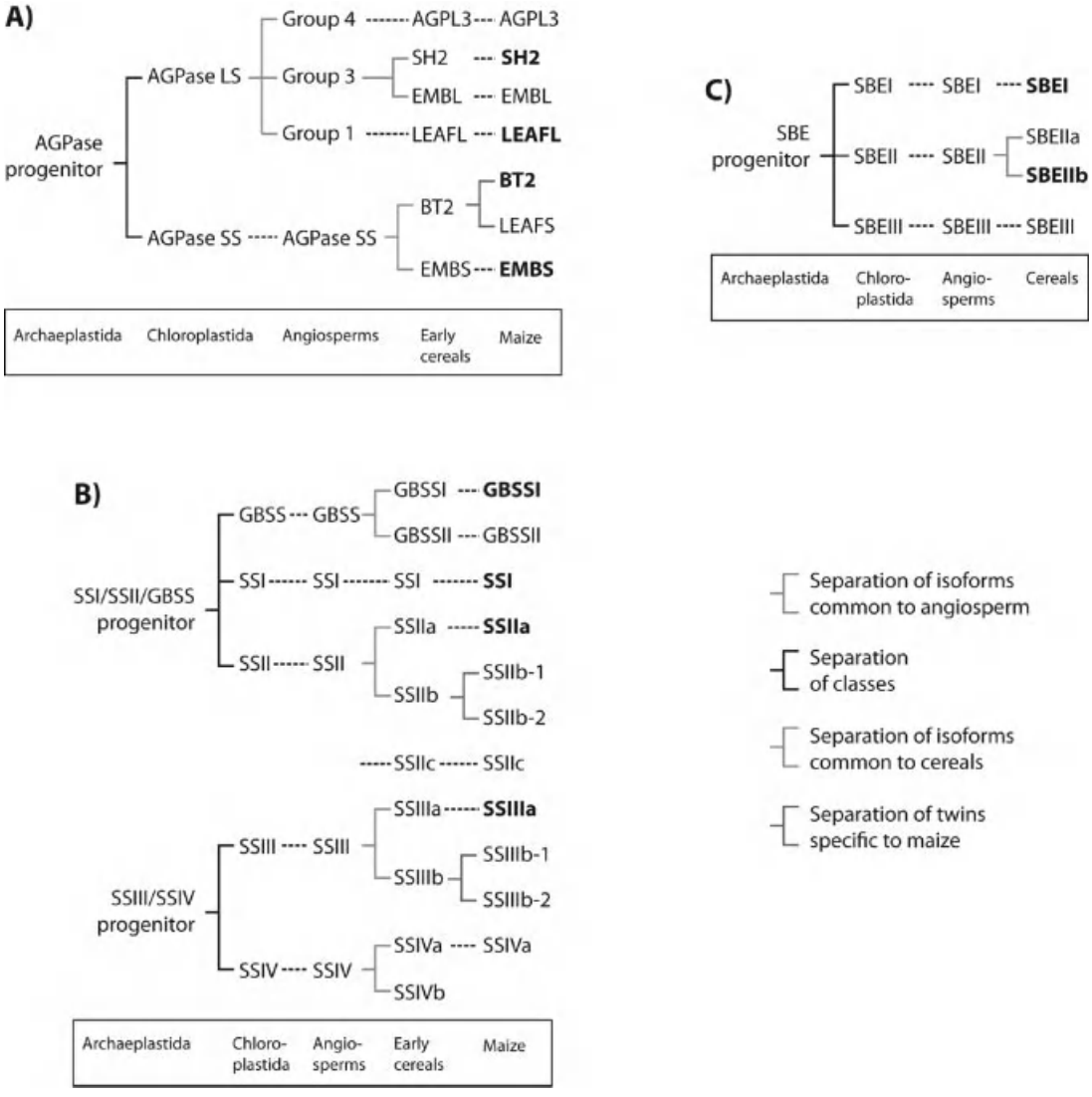

Figure 7.2 Proposed evolution of maize starch biosynthetic genes. Enzymes in bold text have been shown by genetic analysis to be involved in endosperm starch biosynthesis. Only lineages that lead to genes extant in maize are shown. *A)* AGPase. A single gene in the Archaeplastida progenitor, derived from a cyanobacterium, duplicated to form AGPase LS and AGPase SS that are conserved in all chloroplast-containing species. An angiosperm progenitor underwent further duplications to generate a minimum of three isoforms of AGPase LS and one of AGPase SS before separation of monocots and eudicots. In the Graminacea, a whole-genome duplication resulted in two isoforms of the group 3 AGPase LS and two isoforms of AGPase SS. A whole-genome duplication generated the duplicate gene encoding LEAFS that is present in maize but not in rice. *B)* SS. Two genes were present in the primordial Archaeplastida progenitor before divergence of plastid-containing organisms. At the establishment of the Chloroplastida, these had duplicated to form five SS classes, each of which is extant in all green algae and land plants. In the Graminacea, a whole-genome duplication resulted in isoforms of GBSS, SSII, and SSIII, each of which is present in maize and rice. SSIV was also duplicated at this stage, but one copy was not retained in maize. SSIIc could have originated either in an ancestor common to both monocots and eudicots or after that division. A whole-genome duplication generated twin genes for SSIIb and SSIIIb that are present in maize but not in rice. *C)* SBE. Three classes of SBE had been formed before divergence of green algae and land plants by duplication of an ancestral gene. In general, all three classes are conserved in Chloroplastida species, although at least one exception is known. The ancestral whole-genome duplication in the Graminaceae gave rise to two isoforms of the SBE II class, both of which are present in maize and rice. (Adapted from Rosti and Denyer [2007]; Deschamps *et al.* [2008]; Georgelis *et al.* [2008]; and Yan *et al.* [2009a, 2009b].) (For color detail, see color plate section.)

Starch Synthase

Five SS classes are encoded in the maize genome, each of which is conserved in all known chloroplast-containing species (Table 7.1) (Deschamps *et al.*, 2008). Genes exist that encode two isoforms of the exclusively granule-bound enzyme, GBSS, referred to as GBSSI and GBSSII. Similarly, genes encoding three isoforms of the SSII class (SSIIa, SSIIb, and SSIIc) and two isoforms of the SSIII class (SSIIIa and SSIIIb) are present. Twin genes exist in maize for SSIIb (SSIIb-1 and SSIIb-2) and SSIIIb (SSIIIb-1 and SSIIIb-2). The SSI and SSIV classes in maize are encoded by single genes. The amino acid identity between isoforms of the same SS class ranges from 68%–81% over the catalytic domain, whereas the identity between classes ranges from 27%–49%. Classical mutations that condition scorable kernel phenotypes are known in the genes encoding GBSSI, SSIIa, and SSIIIa. From these data and further characterizations of the effects of the mutations on starch structure, it is evident that GBSSI, SSIIa, and SSIIIa all function in starch biosynthesis in endosperm. To date, mutations have not been characterized by either forward or reverse genetics in the maize genes encoding SSI, SSIIb-1, SSIIb-2, SSIIc, SSIIIb-1, SSIIIb-2, SSIV, or GBSSII. Whether or not these other SS enzymes contribute to endosperm starch biosynthesis is unknown. However, the genetic analyses indicate that none of them can fully compensate for loss of GBSSI, SSIIa, or SSIIIa.

Each conserved SS class appears to function in the generation of certain ranges of linear chain lengths within the glucan polymers in starch granules (Jeon *et al.*, 2010; Tetlow, 2011). GBSSI, encoded by *waxy1* (*wx1*), functions specifically to generate amylose. Mutations in *wx1* that prevent GBSSI expression condition complete loss of amylose, without affecting amylopectin structure or overall granule morphology. These data indicate that amylopectin is the key determinant of the semicrystalline nature of starch and of granule structure. Genetic analysis showed that SSIIa, encoded by *sugary2* (*su2*), functions in the generation of medium-length chains within amylopectin. In *su2* mutants, medium-length linear chains of degree of polymerization (DP) 16–24 are decreased in frequency and short chains of DP 8–12 are more abundant relative to wild-type. From these data, SSIIa is thought to elongate pre-existing chains in the size range of DP 8–12. This structural alteration of amylopectin is conserved in many species when SSIIa is mutated. SSIIIa, encoded by *dull1* (*du1*), appears to serve a complex role in determining amylopectin structure (Lin *et al.*, 2012). Chain length profiles show specific decreases of DP 9, DP 17, and DP 18, with increased abundance of DP 11–15 chains. Other reproducible alterations occur between DP 21 and DP 30. This pattern is highly reproducible in mutant cereal endosperm in other species including rice and barley. The effects of *du1* mutations are difficult to explain based on substrate preference for a specific chain length range. SSIII has been proposed to affect the activities of other SSs, in addition to possessing its own catalytic activity. Supporting this hypothesis, SSIIIa has been shown to be present in a high-molecular-weight complex that also contains SSIIa (Hennen-Bierwagen *et al.*, 2009).

Gene expression patterns for the SSs in maize can best be examined by surveying the publicly available RNA-seq data (www.maizegdb.org). In general, there appears to be isoform specificity that agrees with the genetic identification of enzymes required in the endosperm. For classes with multiple isoforms, at least one is expressed predominantly in endosperm, whereas the mRNAs encoding the others are most abundant in nonendosperm tissues. Endosperm-predominant expression is evident for SSIIa, SSIIIa, and GBSSI, encoded by *su2*, *du1*, and *wx1*, in agreement with genetic data indicating roles in storage starch biosynthesis. The relative levels of mRNA accumulation as estimated from RNA-seq data vary greatly for different SS classes and isoforms. The most abundant are the mRNAs encoding GBSSI and SSI, which are present at frequencies close to those of the SH2 and BT2 mRNAs. At the low end of the scale are the SSIIb-2, SSIIc, SSIIIb-1, and SSIV transcripts,

which are observed ~100 times less frequently than GBSSI or SSI in maize endosperm. At this time, no RNA-seq data exist for SSIIb-1 or SSIIIb-2 in the maize genome database, and their roles remain unknown.

From genetic data in other species, particularly rice and *Arabidopsis*, SSI is thought to be responsible for generating short linear glucans within amylopectin of DP 6–10, which are elongated by SSIIa (Delvallé *et al.*, 2005; Fujita *et al.*, 2006). SSIV function has been studied in *Arabidopsis* leaf and potato tuber, where it is thought to influence granule initiation but does not affect amylopectin fine structure (Roldán *et al.*, 2007; Gamez-Arjona *et al.*, 2011). Discerning whether these same functions are provided by SSI and SSIV in cereal endosperm requires further investigation. As with the other SS classes, both SSI and SSIV are conserved in all green plants, and both are expressed at the level of mRNA accumulation in maize endosperm, so functions in seed starch biosynthesis are likely.

The *Archaeplastida* progenitor of green algae and land plants appears to have contained two separate genes encoding SS, which may have been derived from distinct prokaryotic sources (Price *et al.*, 2012). Phylogenetic analyses show clearly that genes encoding each of the five SS classes were present early in *Chloroplastida* evolution, before divergence of green algae and land plants (Figure 7.2*B*) (Deschamps *et al.*, 2008; Yan *et al.*, 2009a, 2009b). The whole-genome duplication that occurred early in the monocot lineage gave rise to the SSIIa/SSIIb, SSIIIa/SSIIIb, and GBSSI/GBSSII isoform pairs, as shown by the presence of orthologs encoding all of these proteins in both maize and rice. The late whole-genome duplication in the maize lineage generated the twin pairs SSIIb-1/SSIIb-2 and SSIIIb-1/SSIIIb-2. The evolutionary history leading to SSIIc is unclear because the duplication that gave rise to this isoform may have occurred either before the split of monocots and eudicots or specifically in the monocot lineage.

Starch Branching Enzyme

Survey of the maize genome reveals four genes that encode SBE (Table 7.1). Biochemical and genetic characterization of plant SBEs has revealed three different enzymes, termed SBEI, SBEIIa, and SBEIIb, which form two conserved enzyme classes. The SBEII class exhibits two highly homologous isoforms. A third conserved class, SBEIII was revealed by survey of the rice, *Arabidopsis*, and poplar genome sequences (Han *et al.*, 2007), and a gene encoding SBEIII also is present in maize. Genes encoding SBEII and SBEIII are present in all plant genomes analyzed, as revealed by sequence identities on the order of $\geq$70% when proteins are compared between species. SBEI genes are nearly universally present in plants; however, this class is not found in *Arabidopsis*. Among all of the genes encoding SS, SBE, or DBE, the lack of a SBEI-encoding gene in *Arabidopsis* is the only example known where one of the conserved enzyme classes is not encoded within a plant genome.

As seen within the SS classes, SBEs also demonstrate substrate specificities for chain length selection and transfer (Guan and Preiss, 1993; Takeda *et al.*, 1993; Guan *et al.*, 1997). *In vitro* assays showed that SBEI has a preference to transfer longer linear chains than those transferred by SBEIIb. The range of chains transferred by SBEIIb is less than DP 10, with the most abundant product branch being DP 6–7. SBEI, in contrast, transfers chains greater than DP 10. In addition, the length of the substrate chain being acted on needs to be longer for SBEI, greater than DP 16, whereas SBEIIb can cleave linear regions of DP 12 or less. SBEIIa has not been characterized by *in vitro* assays; however, the extremely high similarity to SBEIIb suggests the properties of the two isoforms will be similar.

Microarray and deep sequencing data reveal that the genes encoding SBEI, SBEIIa, SBEIIb, and SBEIII all are expressed in endosperm at the level of mRNA accumulation, at varying levels. The maize gene *amylose extender* (*ae*) encodes SBEIIb, which is expressed in endosperm at the highest level among these genes and has a major role in the determination of amylopectin structure. Mutation of *ae* results in a decrease in starch content of ~20% compared with wild-type and causes a major change in amylopectin structure such that the branch linkage frequency is decreased to a large extent and the average linear chain length is increased. SBEIIa does not compensate for loss of SBEIIb, despite the facts that the two proteins are >90% identical and that SBEIIa mRNA is present in endosperm.

Mutations of *sbe2a*, encoding SBEIIa, do not condition changes in endosperm starch content or amylopectin chain length distribution. However, SBEIIa transcript is present in endosperm at about 20% of the frequency of the SBEIIb mRNA. SBEI transcript also is present in endosperm at appreciable levels. Loss-of-function mutations in *sbe1* have a subtle effect on the structure of amylopectin, particularly with regard to the density of branches at the root of crystalline clusters (Yao *et al.*, 2004; Xia *et al.*, 2011). In rice, mutation of SBEI caused slight but reproducible increases in the frequency of short chains of less than DP 10, and slightly decreased frequency of chains of DP 12–21 and greater than DP 37 (Satoh *et al.*, 2003). These data show that SBEI affects endosperm starch structure in cereals. Transcripts encoding SBEIII are present at exceedingly low levels throughout the plant. Discerning whether or not this gene functions in starch biosynthesis in endosperm or any tissue would require loss-of-function mutations.

SBEIIb and SBEI transcripts accumulate predominantly in the endosperm, and both proteins function in seed starch biosynthesis. SBEIIa transcripts are also present in endosperm, but a function for this gene has been demonstrated only for leaf tissue. SBEIII has very low expression, and its function is unknown. Phylogenetics indicates that genes encoding SBEI, SBEII, and SBEIII all were present at the inception of the *Chloroplastida* lineage (Figure 7.2*C*) (Deschamps *et al.*, 2008; Yan *et al.*, 2009b). The SBEIIa/SBEIIb pair of isoforms is present in maize and rice, indicating they arose from the early whole-genome duplication in a cereal ancestor.

Starch Debranching Enzyme

Involvement of DBEs in starch biosynthesis was demonstrated by genetic analyses based on the "phytoglycogen-accumulation" phenotype. Loss-of-function mutations, in particular DBE classes, condition this phenotype, which entails major decreases in starch content; altered amylopectin structure; severely abnormal granule morphology; and appearance of a water-soluble glucan, termed phytoglycogen, that is similar in structure to glycogen from nonplant species (Hennen-Bierwagen *et al.*, 2012). Appreciable amounts of phytoglycogen are not detected in wild-type plants but only in plants with compromised DBE function. In addition to maize, this phenotype has been seen in rice and barley endosperm, *Arabidopsis* leaf, potato tuber, and *Chlamydomonas* cells, indicating the conserved nature of DBE function in starch biosynthesis.

Four classes of DBE are conserved in plants and green algae. One class is referred to as a "pullulanase-type DBE" (PUL), and three classes are referred to as "isoamylase-type DBEs" (ISA). Two different bacterial genes, currently extant in separate prokaryotic species, appear to be the progenitors of the four conserved plant genes. In addition to amino acid sequences, PUL and ISA differ by substrate specificity. PUL enzymes readily hydrolyze $\alpha(1\rightarrow6)$ glycosidic bonds in pullulan, a linear polymer with the structure -(Glc$\alpha(1\rightarrow4)$-Glc$\alpha(1\rightarrow4)$-Glc$\alpha(1\rightarrow6)$)-n, whereas they are inactive or have low activity toward glycogen. ISA enzymes, in contrast, are inactive

toward pullulan but readily hydrolyze branch linkages within glycogen. The primordial ISA gene in plants apparently was amplified and diverged to generate ISA1, ISA2, and ISA3 very early in the evolution of chloroplast-containing organisms, before the separation of green algae and land plants. ISA1, ISA2, and ISA3 are separate classes of enzyme, each with its own conserved function, rather than isoforms that arose as the result of relatively recent gene duplications (Deschamps *et al.*, 2008).

Three genes encoding ISA and one gene encoding PUL are present in the maize genome (Table 7.1). All of the DBEs are members of a structural superfamily of proteins that includes α-amylases, and highly conserved catalytic residues of that family are generally present in the maize DBEs. The exception is ISA2, which has substitutions at multiple positions where catalytic residues are present in other members of the family. These changes render ISA2 noncatalytic for DBE activity. Despite this characteristic, however, the noncatalytic DBE class is highly conserved, indicating a function for ISA2 other than a direct enzymatic role (Hussain *et al.*, 2003).

Genetic evidence indicates function of ISA1 and ISA2 in endosperm starch biosynthesis to varying extents (Hennen-Bierwagen *et al.*, 2012). ISA1 is a key determinant of endosperm starch structure and content, as shown by the fact that mutations of the *su1* gene condition the phytoglycogen-accumulation phenotype. Loss of ISA1 also conditions phytoglycogen accumulation in rice and barley endosperm as well as *Arabidopsis* leaves and potato tubers. In contrast, loss of ISA2 has only small effects on total endosperm starch accumulation or amylopectin structure. Loss of ISA2 in maize endosperm did affect granule size during development, although by maturity the effect was relatively minor and much less severe than granules that form in *isa1-* mutants. Despite this small effect of *isa2-* single mutations, when coupled with loss of SSIIIa or with a partially functional allele of the ISA1-encoding gene, the ISA2 deficiency conditioned a phytoglycogen accumulation phenotype (Lin *et al.*, 2012). Thus, ISA2 in maize endosperm is a major determinant of starch content and structure in some, but not all, conditions.

In contrast to endosperm, ISA2 by itself is required for normal starch content in *Arabidopsis* leaves and potato tubers (Zeeman *et al.*, 1998; Bustos *et al.*, 2004). Deficiency of ISA2 in either instance causes the same phytoglycogen accumulation phenotype as mutations eliminating ISA1. This is explained by the fact that ISA1 and ISA2 function together in a heteromeric enzyme complex, and both proteins are requisite for enzyme activity in *Arabidopsis* or potato. Endosperm differs because ISA1 is enzymatically active either as a homomultimer or as a heteromultimer, and the former can sustain starch biosynthesis at normal levels. The reason for the difference between endosperm and the other tissues is unknown but could be explained either by inherent differences in ISA1 structure between the cereals and other plants or by metabolic or regulatory distinctions between the tissue types.

PUL appears to have a minor effect on starch content in endosperm. Single mutants lacking this enzyme had no discernible effect on starch content or structure. However, when coupled with a partially functional allele affecting ISA1 that does not cause a noticeable phenotype, the double mutants accumulated phytoglycogen (Dinges *et al.*, 2003). This result indicates that PUL can contribute to starch biosynthesis, although it cannot fully substitute for ISA1 as shown by the strong phytoglycogen accumulation phenotype of ISA1 loss-of-function mutations. ISA3 deficiency in endosperm has been analyzed only in rice, where the mutation had minor effects on amylopectin structure without causing reduced starch content.

Transcript encoding ISA1 is the most abundant among the DBE genes in endosperm, in excess of ISA2, PUL, or ISA3 transcripts by factors of threefold to eightfold. The ISA1 transcript is much more abundant in endosperm than in any other tissue; however, the low level seen in other tissues is close to that of ISA2, PUL, and ISA3 (www.maizegdb.org). These data are consistent with ISA1 being the predominant DBE activity involved in starch biosynthesis in endosperm. In summary, ISA1

is the major DBE active in endosperm starch accumulation, ISA2 is not normally required but does contribute a major function when other starch biosynthesis enzymes are defective, PUL provides a minor DBE biosynthetic function in this tissue, and ISA3 appears not to function in this process.

Genes encoding each DBE class were present in the progenitor of the green algae and land plants, as shown by the presence of four paralogs in any genome analyzed to date (Hennen-Bierwagen *et al.*, 2012). Isoforms within classes have not been found, so subsequent to any whole-genome duplication in the cereals one of the two copies has been lost.

Conclusion

The catalytic functions that participate in starch biosynthesis are provided by multiple conserved classes of enzymes. Each species and tissue has at least one SSI, SSII, SSIII, GBSS, and SSIV enzyme present, and similar class conservation is evident for SBE, DBE, and AGPase. Within each of the AGPase, SS, and SBE classes, multiple isoforms have arisen that appear to exhibit specific individual functions. A general conclusion is that one set of isoforms in a class functions primarily in endosperm, and a second set encoded by different genes functions in leaves and other starch-accumulating tissues. The major isoforms in maize endosperm are SSIIa, SSIIIa, GBSSI, and SBEIIb.

In contrast, some classes of starch biosynthetic enzyme exist as single entities. These include all of the DBEs, SSI, SSIV, SBEI, and SBEIII. For these enzyme classes, differences in function between the storage starch pathway in endosperm and transient starch biosynthesis in other tissues may be controlled by regulatory mechanisms specific to each tissue. One example is the DBE function provided by ISA1 and ISA2, where different assemblies of the heteromultimeric enzyme and the ISA1 homomer are evident in leaf and endosperm, even though the same genes are active in both tissues (authors' unpublished results). In the other instances, paralogous genes appear to provide tissue-specific functions.

Questions remain regarding why certain enzyme classes have not duplicated during angiosperm evolution when so many others have evolved to generate multiple isoforms, and further work is necessary to investigate this topic. A hypothesis to explain the divergence of isoforms within many enzyme classes in maize and other cereals is that endosperm-specific functions provide a strong selective advantage to seeds for reproductive fitness. The advent of large starch granules in endosperm that can persist throughout seed development and dormancy may provide such an advantage. Specialized gene functions that generate such granules, rather than the much smaller transient starch granules in leaves and other tissues, may have arisen through the gene duplication and specialization process that has occurred in the cereal lineage.

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8 Evolution, Structure, and Function of Prolamin Storage Proteins

David Holding and Joachim Messing

Introduction

In this chapter, we describe the origin and function of prolamin proteins, which have arisen by divergent evolution in cereal species into multiple subfunctionalized families. At this juncture, we know the positional information of prolamin genes on chromosomes of several cereals because of their sequenced genomes. However, with respect to the overlapping functions of different prolamins in their deposition mainly in the endoplasmic reticulum (ER) and, to a small extent, within storage vacuoles, most advances have been made in maize, rice, and sorghum. In particular in maize, more recent findings have taken advantage of newly developed methods for characterizing existing and new opaque endosperm mutants as well as our ability to eliminate selectively expression of whole subfamilies of prolamins with RNA interference. Upgraded tools for cell biology such as antibody production and cryofixation and freeze substitution for immunogold labeling have also given new insight into high-definition prolamin storage in the ER. Given these advances in the understanding of the genomics and cell biology of prolamins, an overarching review is timely.

Prolamin Multigene Families

Protein Classification

Seed proteins such as those of maize were mentioned in 1821 by J. Gorham but were investigated in more detail by Osborne (1924) using his novel fractionation techniques. They are classified by their solubility largely into water-soluble globulins and alcohol-soluble prolamins. Most cereal crops have a preponderance of prolamins, named for their high percentage of the amino acids proline and glutamine, because they are a major sink for assimilated nitrogen produced during photosynthesis. Cereals have a large endosperm, which persists at maturity. The endosperm arises from a second fertilization of the central cell and consists of two maternal and one paternal chromosome set. The endosperm becomes terminally differentiated, and during germination all nutrients are used to nurse the growing seedling, which arises from the embryo proper. Synthesis of prolamins in the endosperm begins around 10 days after pollination (DAP) and peaks about 18 DAP.

Prolamins are a mixture of proteins of different sizes and composition. Their composition differs among different lines of the same species as exemplified in maize (Wilson, 1989). Their initial

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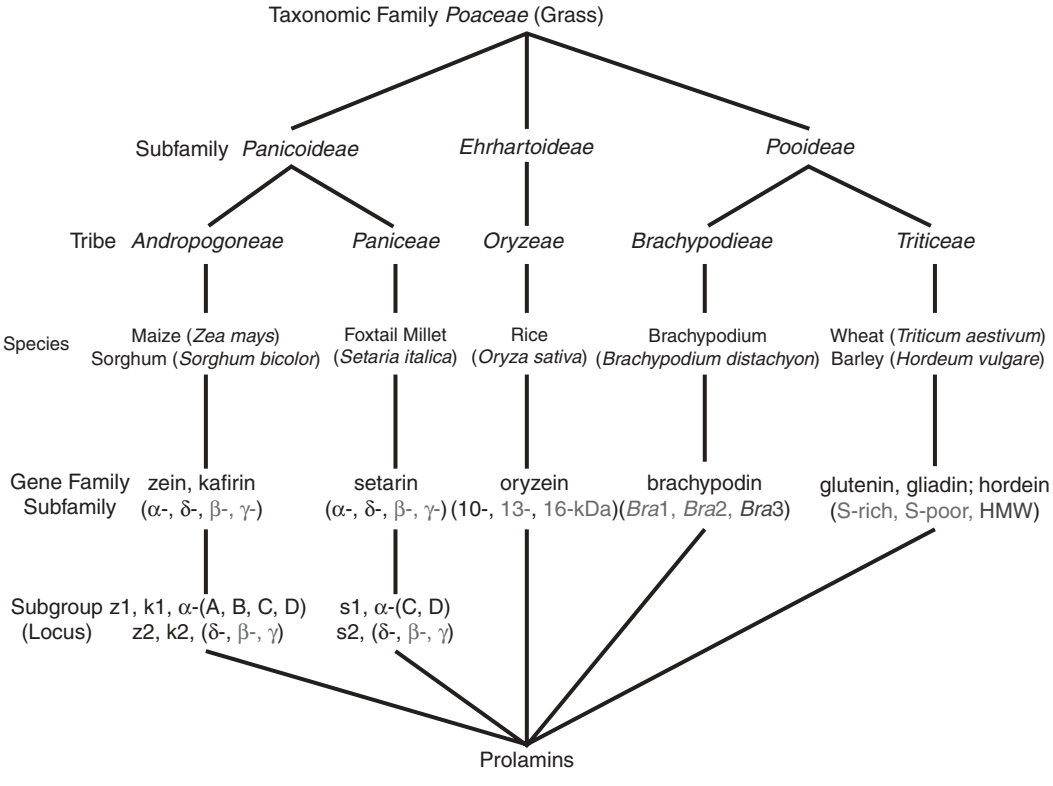

Figure 8.1 Taxonomic relationships of grass species and their prolamin genes. Classification of the subfamilies of grasses that constitute the most important cereals is shown with the different classes of prolamins. (Reproduced from Xu JH, Bennetzen JL, Messing J [2012]. Dynamic gene copy number variation in collinear regions of grass genomes. Mol Biol Evol 29: 861–871.).

classification was based on their mobility in SDS-PAGE under reducing and nonreducing conditions because many of them contain intermolecular cysteine linkages such as the high-molecular-weight (HMW) glutenins in wheat. HMW glutenins are crucial for the strength and elasticity of dough, and the smaller gliadins are crucial for the viscosity and extensibility of dough, illustrating their distinct physical properties (see Chapter 10) (Shewry and Halford, 2002). In maize, prolamins probably reached the greatest diversity of the sequenced grass genomes (Figure 8.1) and were grouped into α-zeins with 22-kDa and 19-kDa, β-zeins with 15-kDa, γ-zeins with 50-kDa, 27-kDa, and 16-kDa, and δ-zeins with 18-kDa and 10-kDa relative molecular weights. Although proteins with similar sizes could be separated by charge with isoelectric focusing, a systematic inventory in each species even at the cDNA level was difficult because of the differential expression of genes in different genetic backgrounds. Such an effort had to wait for the global analysis of entire genomes.

Evolution of Prolamins

To ensure the detection of all gene copies for each storage protein, it became important to construct redundant genomic libraries so that genomic regions could be isolated that contained tandem arrays

of gene copies. Genomic regions also provided us with linked genes. They play an important role for alignment of syntenic regions from closely related species, such as those from taxonomic different subfamilies. The purpose of such alignment is to investigate insertions and deletions of gene copies. Nucleotide substitution rates of gene coding regions can be used to reconstruct the chronology of duplication events.

Such studies illustrate that across syntenic chromosomal regions of different subfamilies of the grasses such as the Pooideae (including wheat, barley, and *Brachypodium*), *Ehrhartoideae* (including rice), and Panicoideae (including Foxtail millet, sorghum, and maize), a copy of a globulin storage protein was contained in a segment of colinear genes. In contrast, the copy of the HMW glutenin storage protein was colinear only in the Pooideae and was missing in the syntenic regions of the Ehrhartoideae and Panicoideae. Phylogenetic analysis of prolamins of all the above-mentioned species indicates that this linked HMW glutenin in wheat is probably the most ancient prolamin gene copy. Given these features, it appears that water-insoluble storage proteins probably arose from water-soluble ones by unequal crossing over and were copied and inserted into other chromosomal locations. In the Pooideae, the new prolamin gene was retained in its original position adjacent to the globulin gene, whereas in the Ehrhartoideae and Panicoideae, the donor copy was deleted (Xu and Messing, 2009).

An interesting aspect of the two different modes, where the donor is retained or not, is the possible slower pace of divergence in the presence of gene conversion because HMW glutenins are present in different loci of wheat, whereas they are completely absent in the *Ehrhartoideae* and *Panicoideae*. These subfamilies instead have γ-prolamins that are closer to the gliadins in wheat, which are in yet different chromosomal locations from the glutenins. This mechanism of dispersal of gene copies is an important mechanism in the divergence of protein structure. A practical consequence is the unique property of wheat with baking quality for various food products such as noodles, unleavened breads, and most importantly leavened bread that is absent in other cereals (see Chapter 10) (Shewry and Halford, 2002). This structural differentiation of prolamins also gave rise to differential human adaptation, where 1% of Northern Europeans and an equivalent number in other populations have developed an autoimmune response against epitopes of certain wheat prolamins but not against the ones from rice or maize (Tye-Din *et al.*, 2010). All prolamins have arisen by internal duplication of a block of amino acids characterized by glutamine residues, which became already apparent when the first prolamin was sequenced (Geraghty *et al.*, 1981). It is the variability of these repeats that provides enormous numbers of epitopes, some that trigger diseases such as celiac sprue but are crucial for the physical properties of flour.

Although HMW glutenins were lost in the *Panicoideae*, they appeared to have generated a distinct new group, the α-prolamins and δ-prolamins (Figure 8.1). The latter ones are a reservoir for methionine, an essential amino acid, and are crucial for the composition of animal feed. The relatively low level of δ-zeins in feed corn requires the addition of chemically synthesized methionine, and this represents a billion-dollar supplement market to support U.S. meat production. In contrast to the δ-zeins, in which each locus comprises a single-copy gene, α-zeins are tandemly amplified in most chromosomal locations. This mode seems to be preserved within the subfamily of the *Panicoideae* as seen in the genomes of Foxtail millet, sorghum, and maize, which belong to the different tribes of the Paniceae and Angropogoneae. Intertribal syntenic alignments have also shown that dispersal occurred before and after tribal split. The oldest copy of the α-prolamins was retained in sorghum but deleted in Foxtail millet and maize. Deletion appears to have occurred through unequal crossing over of two flanking cytochrome P450 gene copies that are still present in sorghum but only as a single copy in Foxtail millet and maize with different crossover points in each species (Xu *et al.*, 2012).

Besides unequal crossing over and dispersal of gene copies into different locations, genes can also be duplicated by whole-genome duplication (WGD). Examples are γ-zeins and δ-zeins. In contrast to sorghum, maize underwent WGD about 4.8 million years ago by hybridization of two diverged progenitors that split from the progenitor of sorghum 11.9 million years ago (Swigonova *et al.*, 2004). After the split, one of the progenitors acquired an α-zein gene copy in a new chromosomal location about 7 million years ago, whereas the other one did not. When syntenic chromosomal regions from the two homeologous regions of the maize genome are aligned with sorghum, this locus is unique to one region in maize, which became maize chromosome 7S. There are also examples where dispersal occurred after WGD, which are also unique to one homeologous region on chromosome 4S. Yet another example is the oldest α-zein locus, which also had to be duplicated during WGD but is also present only in one homeologous region on maize chromosome 1 that is syntenic with sorghum chromosome 8. Because it was present before the progenitors of maize hybridized, it was concluded that it was lost after allotetraploidization of maize. Loss of homeologous gene copies has been estimated to be >50% because of selection against homeologous chromosomes during meiosis. The youngest gene copies clearly arose from tandem duplication, and in maize different inbred lines differ by copy number variation (CNV) in different loci (Xu and Messing, 2008).

Differential Expression of Gene Copies and Allelic Variation

Gene amplification contributes not only to the diversification of protein structure and function but also to the differential regulation of gene expression. The characterization of the members of the multigene family in their genomic position becomes essential. Common cDNA libraries and oligonucleotide array technologies have not been able to match individual gene copies with individual transcripts of the α-prolamins because members of recently amplified copies present in a tandem cluster are too conserved to permit the use of conventional techniques. A major insight of refined methods is that amplification also leads to gene silencing either at the transcriptional or at the post-transcriptional level (Miclaus *et al.*, 2011a). Because of the high percentage of glutamine residues in α-zeins, this is not surprising as the most common mutation is a C to T conversion. Glutamine codons are either CAG or CAA and would become stop codons TAG or TAA, which is the case. In one case, a single premature stop codon of an α-zein gene copy in W22, called csf4c5, has an intact allele with a glutamine codon in BSSS53, called azs22;8 (Llaca and Messing, 1998). Transcripts with premature stop codons have been cloned but at a very low frequency, most likely owing to decreased mRNA stability because of abortive translation (Liu and Rubenstein, 1993). Still the most common question that arises is what selective pressure is responsible for retention of silenced gene copies? We can speculate only that the silenced gene pool serves as a reserve that can be readily activated within a single generation. In such a case, we would propose gene conversion as a rapid way to reactivate gene copies (Llaca and Messing, 1998).

There are several interesting aspects concerning the regulation of the α-zein gene copies. First, CNV within maize inbred lines is the basis for a shift in gene expression. Compared with B73, the z1C1 locus in BSSS53 has two extra copies at the 3′ end of the cluster, which are strongly expressed (Song *et al.*, 2001). In hybrids of BSSS53 and B73, their expression is reduced, whereas other copies are enhanced, with a slight dosage effect because of the triploid endosperm, indicating a trans-acting compensation of gene expression, most likely at the post-transcriptional level (Song and Messing, 2003). Second, in other α-zein gene clusters, there is a major shift of expression to the most recently amplified gene copy. When endosperm is cultured, a condition that is known to reverse

epigenetic silencing, the expression of older gene copies can increase, indicating their expression capacity is retained. Expression of the younger copies is reduced, similarly to the compensation effect observed in hybrid crosses described previously (Miclaus *et al.*, 2011a). Third, bisulfite sequencing of the promoter regions exhibits different methylation patterns for each locus. The dispersal of gene copies provides for a mechanism of bringing a gene copy under the influence of a new chromatin structure, which can differ between donor and the new insertion site (Miclaus *et al.*, 2011a). It is also conceivable that because of chromosomal position, different adjacent transposable elements provide fine-tuning of transcriptional regulation of gene expression as it occurred with the paralogous copy of the *P1* gene in maize (Goettel and Messing, 2010). Fourth, the two new 3′ copies at the *z1C1* locus in BSSS53 compared with B73 lost their transcriptional regulation through the O2 transcription factor as they are expressed in the homozygous *o2* mutant despite the conservation of their promoter regions (Song *et al.*, 2001). It appears that tandem amplification can provide sufficient position effect on gene expression and adaptation to allow novel transcriptional activation.

There is also CNV among γ-zeins at the 27-kDa zein locus. W64A has a single copy, whereas W22 and A188 have two copies (Das and Messing, 1987). There appear to be also allelic variations. One of the QTLs of Quality Protein Maize (QPM; also discussed with opaque mutants further on) correlates with elevated levels of the 27-kDa γ-zeins, maps close to the locus, and could be an allelic promoter version (Holding *et al.*, 2008). There is another QTL that acts in trans and could be an allele of a regulator that acts on the expression of the 27-kDa zein. Earlier studies already indicated differential transcriptional and post-transcriptional regulation of gene expression of the 27-kDa γ-zein locus (Or *et al.*, 1993).

The correlation of increased levels of the 27-kDa γ-zeins in QPM lines indicates an interesting subfunctionalization of this storage protein gene. If higher levels of the 27-kDa γ-zein can overcome the opaqueness and kernel softness in QPM, suppression of γ-zeins should revert QPM seeds to opacity. When a γ-zein RNAi event is crossed with QPM lines, they revert to opaqueness, indicating hypostasis of the QTLs and confirming that kernel hardness requires a certain level of γ-zeins (Wu *et al.*, 2010). There is an interesting twist to this role of γ-zeins; gamma zeins are the oldest prolamin genes in maize, as described previously, and there is a suggestion that they have played a role in domestication. They appear to be regulated by one of the domestication loci of Teosinte, the prolamin box-binding factor (PBF). In contrast to α-zeins-, β-zeins, and δ-zeins, γ-zeins can be activated in tissue culture (Wu and Messing, 2009). The PBF is concomitantly activated in tissue culture suggesting that PBF is insufficient to activate the younger classes of zeins. This indicates they have acquired additional cis-acting elements for other tissue-specific transcriptional activators, as expected for new versus older gene copies. PBF is suspected as one of the few domestication loci in maize versus teosinte (Jaenicke-Despres *et al.*, 2003).

Endosperm Texture and Storage of Prolamins

Early Insights into Physical Role of Zeins

Prolamins accumulate in subcellular structures, called protein bodies. Although the granular inclusions of protein bodies in the maize endosperm were first noticed in 1885 (Harz, 1885), Duvick first described their growth as cytoplasmic inclusions in 1955. Duvick (1955) described how starch grains were larger and more numerous in the central endosperm cells, and protein granules were larger and more numerous in the peripheral layers. He developed a model (summarized in

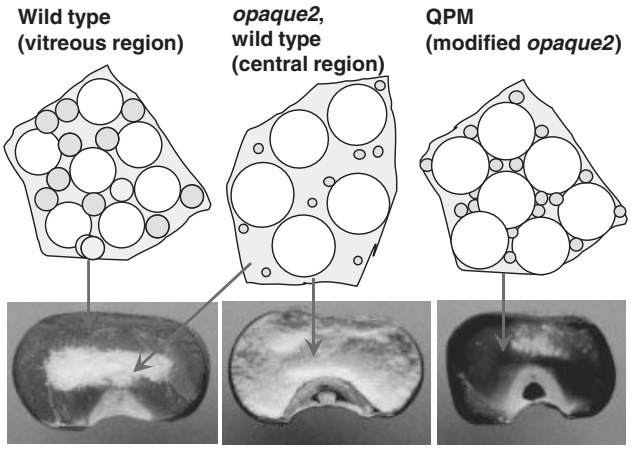

Figure 8.2 Diagram of protein body and starch grain interaction in vitreous endosperm formation. Individual cells of developing endosperm are represented with the relative size and abundance of starch grains (white spheres) and zein protein bodies (gray spheres) that are thought to result in vitreous or opaque endosperm in normal, *opaque2,* and modified *opaque2* (QPM) kernels. (For color detail, see color plate section.)

Figure 8.2) for how their size and distribution relative to that of starch grains is key to the development of the vitreous (hard) endosperm, which is such an essential agronomic trait in corn (Duvick, 1961). Duvick compared the structure of the vitreous endosperm with a box of white marbles (starch grains) densely interspersed with buckshot (protein bodies) and all bound by a transparent glue (clear viscous cytoplasm), which forms a rigid conglomerate when dry (Duvick, 1961). At the boundary between the region that will give rise to the vitreous endosperm and the central starchy (opaque) endosperm, a threshold is reached where the relatively small size of the protein bodies and the large size of the starch grains does not produce a rigid glassy matrix on dehydration but rather a friable chalky structure that is incapable of transmitting light and is opaque (Duvick, 1961). This model (Figure 8.2) and the fact that zein abundance is closely correlated with protein body size is consistent with observations that the vitreous endosperm contains much more zein than the soft central region (Hamilton *et al.*, 1951; Dombrink-Kurtzman and Bietz, 1993). Environmental conditions resulting in reduced zein synthesis, such as nitrogen depletion, result in kernels that are soft and starchy throughout (Tsai *et al.*, 1978).

Formation of ER Protein Bodies in Starchy Endosperm

The differentiation of protein structure during evolution of zein genes also determines their cellular location and contribution to subcellular and seed structures. Zein synthesis begins in starchy endosperm cells at around 8–10 days after pollination (DAP) and continues throughout endosperm development, terminating from the center outward as cells undergo programmed cell death (PCD) (Young and Gallie, 2000). The onset of expression of the different classes of zeins is not uniform (Woo *et al.*, 2001). For example, the γ-zeins are synthesized throughout the endosperm before the onset of α-zein and δ-zein expression, and this is consistent with their predicted role in priming ER-protein body formation (Woo *et al.*, 2001). All zein proteins are initially translated with signal peptides, which are subsequently cleaved after the proteins have entered the lumen of the rough endoplasmic reticulum (rER). Despite the fact that none of the zein proteins contain a canonical ER

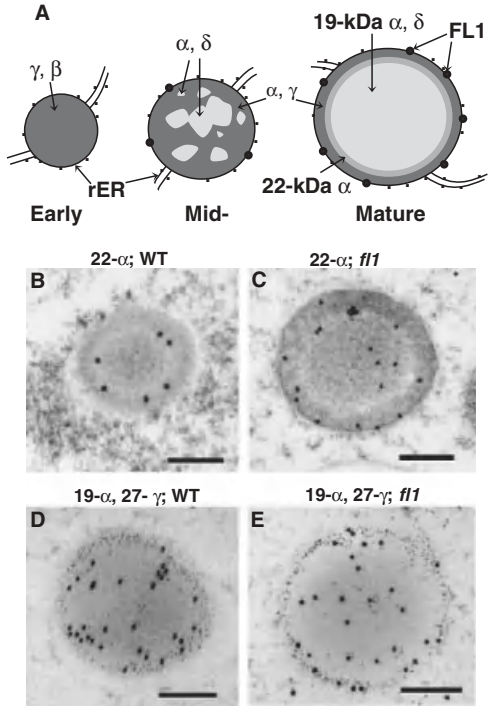

Figure 8.3 Organization of zeins in ER protein bodies. *A,* Diagram of zein distribution in early, mid-, and mature protein bodies. Small black dots in the membrane represent ribosomes, whereas large black dots represent FLOURY1 protein. *B,* Immunogold localization of 22-kDa α-zein in wild-type protein bodies (15-nm gold particles). *C,* Immunogold localization of 22-kDa α-zein in *floury1* mutant protein bodies (15-nm gold particles). *D,* Double immunogold localization of 19-kDa α-zein (15-nm) and 27-kDa α-zein (5-nm) in wild-type protein bodies. *E,* Double immunogold localization of 19-kDa α-zein (15-nm) and 27-kDa α-zein (5-nm) in *floury1* mutant protein bodies.

retention sequence, in the starchy endosperm cells zeins remain in the ER, where they are assembled into organized layered accretions referred to as protein bodies (Figure 8.3). Initially, protein bodies consist entirely of cysteine-rich β-zeins and γ-zeins cross-linked by disulfide bonds (Lending and Larkins, 1989; Lopes and Larkins, 1991). Later, the hydrophobic α-zeins and δ-zeins infiltrate the matrix of β-zeins and γ-zeins as variably sized inclusions (Figure 8.3, *middle panel*). Fully expanded mature protein bodies of 1–2 μm have an even round shape with α-zeins taking up the core surrounded by a shell of β-zeins and γ-zeins (Figure 8.3*A, right panel*). This model, developed from immunogold transmission electron micrographs of chemically fixed endosperm samples, did not distinguish between the 19-kDa and 22-kDa α-zeins (Lending and Larkins, 1989). However, antibodies specific to 22-kDa and 19-kDa α-zeins and cryofixation of endosperm samples revealed that these proteins have distinct patterns of accumulation (Figure 8.3, *right panel*). Although the 19-kDa α-zein is found throughout the protein body core, the 22-kDa α-zein is found only in a discrete ring at the interface between the 19-kDa α-zein–rich core and the 27-kDa γ-zein–rich peripheral region (Holding *et al.*, 2007). This is supported by a demonstrated lack of interaction between the 19-kDa and 22-kDa α-zeins (Kim *et al.*, 2002) and may suggest that a 22-kDa interface between the γ/β zein shell of the 19-kDa α-zein core is a critical structural feature. Specific RNAi suppression of the 22-kDa α-zein leads to blebbing of the protein body surface, perhaps as a result of improperly constrained 19-kDa α-zein (Wu and Messing, 2010a).

In general, investigations of the temporal and spatial patterns of zein gene expression as well as a complete analysis of all classes of zein interactions reinforced the above-described model (Woo *et al.*, 2001; Kim *et al.*, 2002). It is thought that direct interactions between zeins are essential for their retention as properly formed protein bodies (Kim *et al.*, 2002). This thought is supported by experiments that used heterologous expression of both α-zeins and γ-zeins in transgenic tobacco leaves. When α-zeins or δ-zeins were synthesized singly, both proteins appeared to be secreted and become degraded (Williamson *et al.*, 1988; Bagga *et al.*, 1995; Coleman *et al.*, 1996; Bagga *et al.*, 1997). However, if α-zeins or δ-zeins are coexpressed with β-zeins and γ-zeins, significant zein accumulation in the ER occurs (Bagga *et al.*, 1995; Coleman *et al.*, 1996; Bagga *et al.*, 1997). This suggests that β-zeins and γ-zeins are essential for the ER retention of α-zeins and δ-zeins. It was subsequently shown that the PPPVHL proline-rich repeats in the 27-kDa γ-zein N-terminal region are necessary for anchoring it within the ER (Geli *et al.*, 1994). The significance of the 27-kDa γ-zein N-terminal region for ER retention was also shown by the synthesis of a protein chimera called Zeolin, consisting of the 27-kDa γ-zein N-terminal region containing the proline-rich repeats fused to the C-terminal region of a bean phaseolin protein (a 7S globulin) (Mainieri *et al.*, 2004). 7S globulins normally form soluble homo-trimers that leave the ER and are trafficked through the Golgi and finally into storage vacuoles (Chrispeels, 1983). However, as a 27-kDa γ-zein fusion, the phaseolin portion was retained in ER-protein bodies in transgenic tobacco leaves. Corn and bean proteins when consumed together provide an adequate balance of the essential amino acids lysine and tryptophan, which are deficient in cereals, and sulfur amino acids, which are deficient in legume seeds. The zeolin study showed the potential for designer protein storage in the ER fraction (Mainieri *et al.*, 2004). However, it did not take into account that expression of significant amounts of transgenic proteins is usually difficult in seed tissues in the presence of large amounts of endogenous storage proteins (Holding and Larkins, 2008). With modern RNAi technology for reducing the levels of particular proteins, this technology has renewed potential.

The γ-zeins and β-zeins act redundantly in the cross-linked protein body shell, and severely abnormal α-zein protein bodies result from suppression of γ-zeins and β-zeins (Wu and Messing, 2010a). However, because abnormal protein bodies are still ER localized, it is likely that other nonzein factors such as FL1 that are not present in heterologous systems are also essential for ER retention. Other mechanisms besides zein interactions are involved in sequestration of prolamins within ER protein bodies. For example, in rice, differential distribution of mRNAs to different ER regions is thought to be an important prerequisite to protein body formation (Okita and Choi, 2002). Protein chaperones also play an important role. A protein called b-70, which is upregulated in the maize *fl2* mutant (described later), was identified as a homolog of the mammalian immunoglobulin binding protein, BiP (Fontes *et al.*, 1991). BiP drives the ATP-dependent folding of proteins and is itself retained in the ER lumen with a C-terminal HDEL retention sequence. Mutations that cause the aberrant accumulation of zein storage proteins (described later) cause elevation of BiP (Boston *et al.*, 1991). BiP is also demonstrated to be central to prolamin protein body formation in rice endosperm (Li *et al.*, 1993). In addition to BiP, protein disulfide isomerase (PDI) activity is essential to prolamin folding, and the abundance of PDI increases similar to BiP in response to abnormally processed zeins (Fontes *et al.*, 1991; Li and Larkins, 1996). In rice, the Cys-rich 10-kDa prolamin (crP10) is directed to the core of ER protein bodies, whereas the Cys-poor 13-kDa prolamin (cpP13) is directed to the protein body periphery (Kumamaru *et al.*, 2007), suggesting that crP10 sulfhydryl oxidations are necessary for rice protein body formation. Work in rice has shown that there are distinct PDI-like proteins (PDIL1;1 and PDIL2;3) that have nonredundant roles in protein body formation (Onda *et al.*, 2011). PDIL1;1, the ortholog of the PDI protein increased in the maize *fl2* mutant ER (Li and Larkins, 1996), is localized in the ER lumen. When PDIL1;1 is knocked out in

the ESP2 mutant, large protein aggregates, consisting of incorrectly placed intermolecular disulfide bonds, form within the ER (Kawagoe *et al.*, 2009), demonstrating an important role for this protein in oxidative folding of proglutelins. It was shown that PDIL2;3 was unable to complement ESP2 and that PDIL2;3 functions specifically in placement of Cys-rich prolamins such as crP10 in the protein body core. When PDIL2;3 was knocked down, crP10 was no longer directed to the protein body core, and conversely, crp10 knockdown displaced PDIL2;3 to the lumen of the ER from its normal location in the protein body periphery supporting the specific functional interaction.

Insights from Zein Storage within Vacuoles in Aleurone Cells

The primary storage site of zeins is the starchy endosperm tissue whose cells die during maturation leaving a network of starch grains and protein bodies. The outermost endosperm layer, the aleurone, remains living and is responsible for secretion of amylases and proteases into the starchy endosperm during germination. RNA in situ hybridization experiments suggested that zein genes may also be expressed in aleurone cells (Woo *et al.*, 2001), and this was confirmed for all classes of zeins using RT-PCR on aleurone peels (Reyes *et al.*, 2011). However, in the absence of typical ER protein bodies, it was unknown if significant zein protein accumulation occurs. Immunogold transmission electron micrograph studies showed that there is negligible accumulation of 19-kDa α-zein and 27-kDa γ-zein in the aleurone, but significant amounts of 22-kDa α-zein, 15-kDa β-zein, and 18-kDa δ zein are present. These zeins exist in protein storage vacuoles (PSV) and result from the delivery of zeins not retained as ER inclusions. Zeins pass as multivesicular bodies and are incorporated into multilayer autophagic compartments before fusing into PSVs (Reyes *et al.*, 2011). Autophagy and de novo formation of prolamin storage vacuoles also occur in wheat where prolamin inclusions exit the ER and are incorporated into PSVs in the starchy endosperm cells (Levanony *et al.*, 1992). The absence of 27-kDa γ-zein and 19-kDa α-zein in maize aleurone PSVs supports previous data suggesting that zeins have defined and separable roles in ER protein body formation. Because 27-kDa zein is thought to prime ER protein body formation (Woo *et al.*, 2001), 15-kDa β-zein alone may be insufficient for this process. The 19-kDa α-zeins are the most abundant of the zeins and appear to be responsible for the bulk of protein body filling. Perhaps the rapid and extensive filling of the ER lumen with 19-kDa α-zein provides a physical blockage that facilitates the collective retention of all zeins in the ER. In the absence of 19-kDa α-zeins, it appears that the 22-kDa α-zeins alone are unable to expand protein bodies sufficiently to cause ER retention. Aleurone PSVs containing zeins contain intravacuolar membranes that are more prominent at 14 DAP and become less prominent by 22 DAP. These are likely remnants of ER membranes because ER membrane proteins TIP3-4 and FL1 were also detected in aleurone cells (Reyes *et al.*, 2011) and support the proposed specific functional association between FL1 and 22-kDa α-zein. In addition, FL1 likely plays an active role in organizing zein inclusions in aleurone PSVs. This is inferred from expression of FL1-mOrange fluorescent protein in cultured endosperm cells, in which aleurone and starchy endosperm cell types are discernible (Reyes *et al.*, 2010). In the early stages after transformation (6 days), cultured cells showed reticulate FL1 expression, whereas by 8 days, its expression was punctate and appeared to delimit the zein-rich inclusions (Reyes *et al.*, 2011).

Nonvitreous Phenotypes in Maize

Perhaps the best demonstration of the central role zein protein bodies play in vitreous endosperm formation has come from the study of opaque endosperm mutants, of which at least 18 have been

described in maize, excluding the RNAi-mediated ones (Thompson and Larkins, 1993), although less than half have been described at the molecular level. These often have alterations in zein synthesis that result in protein bodies with abnormal morphology, size, or number. One opaque mutant that was described more recently results from a disruption in a gene encoding arogenate dehydrogenase, which is involved in synthesis of the amino acid tyrosine and causes pleiotropic effects on amino acid profiles and a generalized reduction in zeins (Holding *et al.*, 2010). It is clear that factors other than zeins and protein bodies are also involved in vitreous endosperm formation. The interaction of developing starch grains (amyloplasts) and protein bodies may be essential in the model described previously (Figure 8.2). In support of this, the *opaque5* mutant was recently cloned and found to encode monogalactosyldiacylglycerol synthase (MGD1) whose interruption caused a strong reduction of galactolipid abundance in the endosperm (Myers *et al.*, 2011). This resulted in changes in starch production and the appearance of compound starch granules, which could interact differently with zein protein bodies. The study underlined the importance of amyoplast membranes in proper starch granule development. As a further demonstration of the importance of zein-independent processes in vitreous endosperm formation, some of a collection of mutator transposon generated opaque endosperm mutants show no qualitative or quantitative changes in their zein profiles relative to wild-type (D. Holding, unpublished data).

Historically, maize opaque mutants have been intensely studied because of their improved grain protein quality in terms of amino acid composition, which in some cases approaches that of reference animal proteins such as milk. This is driven by reductions in the accumulation the lysine-devoid and tryptophan-devoid zein proteins, which have variable effects on protein body morphology. The *opaque2* (*o2*) and *floury2* (*fl2*) mutants were originally reported in 1935 (Emerson *et al.*, 1935) and contain double the wild-type levels of lysine and tryptophan (Mertz *et al.*, 1964; Nelson *et al.*, 1965). The *O2* gene encodes a transcriptional activator that positively regulates the expression of 22-kDa α-zein genes (Schmidt *et al.*, 1990; Ueda *et al.*, 1992) as well as many other genes (Damerval and Devienne, 1993). The reduction of zeins in *o2* does not change the overall seed protein concentration but results in increased accumulation of non-zein proteins, some of which are relatively rich in lysine and tryptophan, such as eEF1A (Lopez-Valenzuela *et al.*, 2004). At the subcellular level, the main effect of the low level of α-zeins in *o2* is a reduction in protein body number and size (Figure 8.4).

Despite its improved nutritional quality, *o2* was not commercially developed because of the undesirable agronomic properties associated with the soft kernel phenotype, such as impaired harvesting and handling characteristics, increased pathogen susceptibility, and reduced yield. However, through extensive breeding efforts in Mexico (Villegas *et al.*, 1992) and South Africa (Geevers and Lake, 1992), a new type of *o2* mutant called Quality Protein Maize (QPM) was developed in which genetic modifiers restore the vitreous kernel phenotype while maintaining the enhanced nutritional quality (Paez *et al.*, 1969; Ortega and Bates, 1983). As mentioned previously, several studies have shown that the 27-kDa γ-zein is increased twofold to threefold in QPM (Wallace *et al.*, 1990; Geetha *et al.*, 1991; Lopes and Larkins, 1991), and the degree of endosperm vitreousness closely correlates with the level of 27-kDa γ-zein protein (Lopes and Larkins, 1991). The 27-kD γ-zein gene maps to a major QTL for endosperm hardness in QPM (Holding and Larkins, 2008; Holding *et al.*, 2011). Given the evidence that 27-kDa γ-zein initiates protein body formation, it is thought that this increase results in elevation of protein body number in QPM (Lopes and Larkins, 1995; Moro *et al.*, 1995), which increases protein cross-linking and restores kernel hardness (Figure 8.2).

Another soft endosperm mutant, *opaque15* (*o15*), shows a twofold to threefold reduction in 27-kD γ-zein synthesis, resulting in a reduced protein body number but unaltered protein body size, again consistent with the 27-kD γ-zein acting in the protein body initiation (Dannenhoffer *et al.*, 1995).

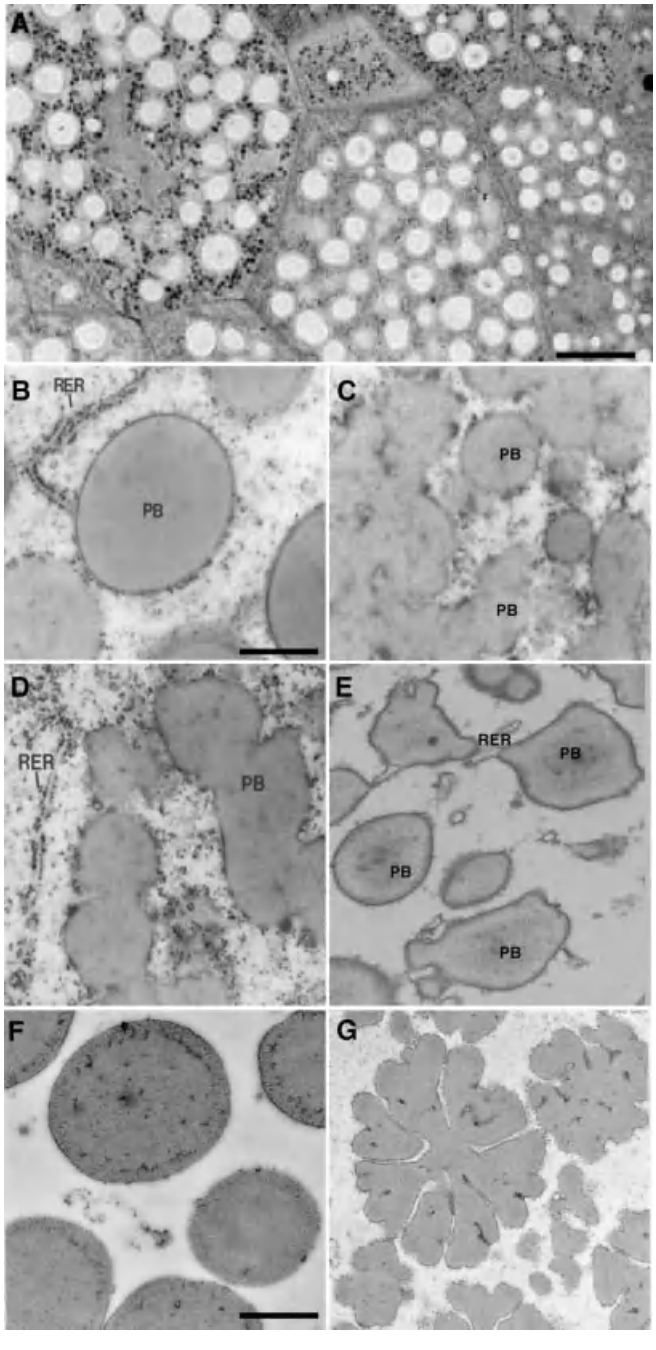

Figure 8.4 Protein body morphology in opaque endosperm mutants. All images are transmission electron micrographs. *A,* Low-magnification transmission electron micrograph section of an *o2-Spmo2o2* mutable kernel showing a boundary region between wild-type cells *(top)* with normal protein bodies and *o2/o2/o2* cells *(bottom)* that have small, less numerous, and faintly staining protein bodies. Scale bar = 50 μm. *B–E,* Protein bodies (PB) in wild-type, *fl2, De-B30,* and *Mc* developing endosperm. RER, rough endoplasmic reticulum. Scale bar in *B* is 200 nm and applies to *B–E*. *F* and *G,* Protein bodies in developing sorghum endosperm of wild-type *(F)* and HDHL mutant *(G)*. Scale bar in *F* is 500 nm and applies to *F* and *G*.

The *O15* gene maps to the same region on chromosome 7L as an *o2* modifier gene (Dannenhoffer *et al.*, 1995) suggesting that *O15* could itself be a modifier gene. In addition to γ-zein, there are several other QPM QTLs, and the extent to which γ-zein is alone sufficient for modification is unknown.

Besides *o2* and *fl2*, a third high-lysine mutant, *o7* arose spontaneously in the W22 background (McWhirter, 1971). Because of its low penetrance in other backgrounds, its molecular basis has remained unknown for a long time despite its importance. However, using transposon mutagenesis in the original background of W22, it has become possible to isolate new alleles of an acyl-CoA synthetase-like gene (ACS), located on chromosome 10L (Miclaus *et al.*, 2011b). ACS could modify the hydrophobicity of proteins so that they could locate into membranes (Black and DiRusso, 2007). The original mutation had just four amino acids in frame deletion, whereas the transposon insertion occurred in the C-terminal region, indicating that mutants might still have residual function. The truncation appeared to have a stronger effect on protein body formation than the in-frame deletion.

The recessive *o2* mutant results from a generalized reduction in α-zeins. The multigenic nature of the α-zein families explains the lack of detection of recessive mutations in the zein genes themselves. However, several dominantly acting mutations in zein genes have been described. The first of these is *fl2*, which was identified as a point mutation resulting in a single amino acid substitution in the N-terminus of a highly expressed member of the *z1C* α-zein gene family (Coleman *et al.*, 1997). This results in failed cleavage of the ER-signal peptide, and a 24-kDa α-zein species is easily identified on SDS-PAGE gels (Figure 8.5). At the cellular level, the abnormal zein is thought to accumulate at the ER membrane rather than getting recruited into the protein body core. The *Defective endosperm B30* (*De-B30*) mutant results from a similar point mutation and amino acid substitution in the ER-signal peptide region, in this case in a 19-kDa α-zein gene (Kim *et al.*, 2004). The dominant phenotype in De-B30 is caused by a mutation in a gene of low-level expression, and the mutant protein could be detected only by immunoblotting (Figure 8.5) (Kim *et al.*, 2004). *fl2* and *De-B30* have protein bodies displaying an uneven, distorted shape (Figure 8.4) (Lending and Larkins, 1992; Kim *et al.*, 2004).

The *Mucronate* (*Mc*) mutant, which also has misshapen protein bodies, results from an abnormal 16-kDa γ-zein in which a 38-bp deletion creates a frame-shift mutation and an abnormal sequence for the last 63 amino acids of the protein (Kim *et al.*, 2006). The mutant protein has a similar length to the wild-type protein but was identified using two-dimensional SDS-PAGE by virtue of its altered isoelectric characteristics. The defective 16-kD γ-zein in *Mc* shows a reduced interaction with 22-kD α-zeins (Kim *et al.*, 2006), which could disrupt the organization of zeins within the protein body. *De-B30*, *fl2*, and *Mc* each result in increased expression of genes associated with

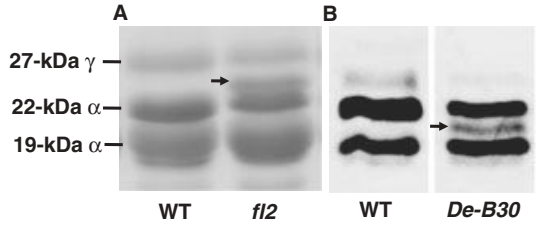

Figure 8.5 Abnormal α-zeins in *fl2* and *De-B30* mutants. *A,* Coomassie blue–stained SDS-PAGE of purified zeins showing high-level accumulation of abnormal 24-kDa α-zein in *fl2*. *B,* Protein gel blot of purified zeins labeled with general α-zein antiserum showing low-level accumulation of abnormal uncleaved 19-kDa α-zein in *De-B30.*

the unfolded protein response (UPR) (Hunter *et al.*, 2002), consistent with the expression of a malfolded, ER-localized protein.

Fl2 and *De-B30* protein bodies both exhibit lobed protein bodies (Figure 8.4), indicating that failure of a percentage of α-zeins to detach from the ER membrane prevents normal separation of discrete-sized ER accretions. However, normal amounts of γ-zeins are likely able to encase much of the α-zein, giving rise to relatively even curved surfaces on the lobed protein body structures. The *Mc* phenotype is distinct from *fl2* and *De-B30* because lobing is not the primary defect but rather more random distortions are the primary defect, often including jagged edges (Figure 8.4). This indicates that the dominant mutant 16-kDa γ-zein may interfere with the collective γ-zein and β-zein function to enclose α-zeins in the protein body core. Immunolocalization studies of each zein species in the *Mc* mutant would allow a better understanding of the cause of this phenotype.

o2 and *opaque 1* (*o1*) (Nelson *et al.*, 1965) also show UPR induction but to a lesser extent than *De-B30*, *fl2*, and *Mc* (Hunter *et al.*, 2002). *o1* has not been cloned and shows no apparent alteration in its zein profile consistent with the fact that vitreous endosperm formation involves both zein-dependent and zein-independent processes.

The *floury1* (*fl1*) mutant was first reported in 1915 (Hayes and East, 1915), but in contrast to *o2* and *fl2*, it was not widely studied because it does not have an improved lysine content. Similar *o1, fl1* does not have reduced accumulation of zeins and lacked an obvious explanation for its kernel phenotype. *FL1* encodes a novel ER protein, which is concentrated in the ER membrane surrounding zein protein bodies (Holding *et al.*, 2007). Apart from the opaque endosperm, *fl1* mutants do not share any of the phenotypes associated with previously described opaque mutants, such as general or specific reductions in zein accumulation; a constitutive UPR, as occurs in *fl2*, *DeB30*, and *Mc*; or alterations in protein body size and shape. The only defect at the cellular level is that *fl1* mutants accumulate 22-kDa α-zeins in abnormal locations within protein bodies. As mentioned earlier, the 22-kDa α-zeins occupy a discrete ring at the interface between the protein body core and the γ-zein–rich periphery (Figure 8.3). In *fl1* protein bodies, 22-kDa α-zein is randomly dispersed and is found in the center of the core as well as in the periphery, often in close association with the ER membrane (Figure 8.3). The 19-kDa α-zeins, which have a more generalized distribution throughout the protein body core, are not altered by the *fl1* mutation (Figure 8.3). Immunolocalizations appear to show increased 22-kDa α-zein accumulation, consistent with a significant increase in quantitative measurements of zein abundance in *fl1* (Holding *et al.*, 2007). These results support the proposed role for FL1 in the correct placement of the hydrophobic 22-kDa α-zeins and suggest that this location is essential for proper protein body formation and the generation of vitreous endosperm but do not rule out its involvement with zein packaging in a more general way.

Natural Variants of Sorghum

The High Digestibility High Lysine (HDHL) mutant of sorghum shares many of the same characteristics as *fl2* and *De-B30*, including an opaque kernel texture (Oria *et al.*, 2000). It has high lysine owing to reduced levels of α-kafirin proteins, which similar to the α-zeins are devoid of this essential amino acid. The molecular nature of the mutation is unknown, but its determination is a high priority because digestibility of the mutant grain is a highly important attribute. Sorghum grain is normally only ∼46% digestible by humans in comparison with ∼73% for maize (Maclean *et al.*, 1981), but digestibility of the mutant grain is increased to ∼85% (Oria *et al.*, 2000). Although α-kafirins make up ∼80% of total kafirins (Watterson *et al.*, 1993), they themselves are not especially recalcitrant

to digestion (Oria *et al.*, 2000). The indigestibility is due to the highly cross-linked nature of the β-kafirins and γ-kafirins (Oria *et al.*, 1995), which normally have a peripheral location enshrouding the α-kafirins in sorghum protein bodies, similar to the arrangement of zeins in maize. Protein bodies in the HDHL mutant have a highly reticulated shape, and the γ-kafirins are found at the base of deep invaginations instead of encircling the protein bodies (Figure 8.4). This likely increases digestibility by increasing the surface area for protease action as well as unmasking the central α-kafirins from the cross linked shield of γ-kafirins (Oria *et al.*, 2000). Although the protein body phenotype is distinct from *fl2*, *De-B30*, or *Mc*, it is not unlikely that it could result from a dominant mutant kafirin, which is consistent with the elevated expression of genes that signify a UPR (B. Hamaker, personal communication). Alternatively, a mutation in an uncharacterized kafirin organizing factor analogous to Fl1 could be responsible.

Structure and Function Analysis with RNA Interference

The progress made in understanding the role of individual zein classes in ER sequestration has been limited for a number of reasons. First, there is a paucity of natural mutants with such phenotypes. One reason is the multigenic nature of the α-zein subfamilies, and the high-level expression of multiple members means that there is substantial functional redundancy (Miclaus *et al.*, 2011a), which would mask the effects of recessive mutants in individual family members. Second, the dominant mutants and *o2* all have pleiotropic effects, which complicate the evaluation of the roles of individual zeins. RNAi technology offers the ability to reduce substantially or eliminate the synthesis of entire subfamilies of zeins (Segal *et al.*, 2003).

Knockdown of 22-kDa zeins at the *z1C1* and *z1C2* loci creates a phenotype that is similar to the *o2* mutation that prevents transcription of the *z1C1* and *z1C2* loci except for CNV of the *z1C1* locus, where CNV could provide a lower penetrance of *o2*. RNAi is not only dominant but also more specific for target genes and more comprehensive. Similar to *o2* mutants, RNAi for *z1C1* and *z1C2* exhibits an opaque phenotype and a higher lysine level. In addition to opaque kernels, the reduction of 22-kDa α-zein in the z1C RNAi as described previously resulted in protein body alterations that were distinct from *o2* (Segal *et al.*, 2003; Wu and Messing, 2010a). Protein bodies were not reduced in size similar to *o2* suggesting that the 19-kDa α-zeins are responsible for the bulk of protein body filling. Small budding protuberances were observed at the protein body peripheries. This supports the suggestion that 22-kDa α-zeins are necessary for properly packaging the 19-kDa α-zeins within the γ-zein periphery.

Mutants such as *fl2*, *De-B30*, and *Mc* are semidominant. These mutations in the coding regions of specific zein genes appear to interfere with α-zein protein accumulation in *trans* leading to generalized zein shutdown as a result of an unfolded protein response. If this is the case, RNAi against a transcript of a mutated coding region should suppress the dominance of such mutations. A construct that simultaneously knocked down the related 27-kDa and 16-kDa γ-zeins prevented the expression of the *Mc* mutation, which contains a frame shift in a 16-kDa γ-zein. Because of the frame shift, the carboxy terminus of the protein has different physical properties to the native protein and is suspected to trigger the UPR. No UPR is detected when the RNAi transgene is combined with the *Mc* mutation (Wu and Messing, 2010b).

To discriminate the roles of the γ-zeins and β-zeins, which are both found in the protein body periphery, another RNAi line was made that targeted the 15-kDa β-zein (Wu and Messing, 2010a). Individually, both of these RNAi lines against the γ-zeins and β-zeins had only minor effects, slightly increasing the proportion of small, unexpanded protein bodies relative to wild-type. However, when

the two transgenes were combined, plants that had substantial reductions in both γ-zeins and β-zeins exhibited irregular-shaped protein bodies, possibly indicating a threshold requirement (Wu and Messing, 2010a). This might suggest that severity of phenotype has a simple correlation with the amount of zein, which is reduced. Although γ-zeins represent the second most abundant class of zeins after α-zeins, it may be that only when both γ-zeins and β-zeins are knocked down is the zein reduction in the protein body periphery substantial enough to cause major effects. This additive effect shows that γ-zein and β-zein act with some degree of redundancy. Although not as severe, their combined affect is reminiscent of *Mc* mutants and suggests that the irregularity results from improperly constrained α-zeins. When the γ/β RNAi was combined with the z1C RNAi, severely lobed and reticulated protein bodies resulted (Wu *et al.*, 2010). This suggests that the outer γ/β layer and the intermediate 22-kDa α-zein sublayer both participate in constraining the abundant 19-kDa α-zeins. Because the γ-zeins and β-zeins are rich in cysteine, they also play a role as a sink for reduced sulfur (see later).

A double RNAi against 22-kDa and 19-kDa zeins reduces α-zein expression to lower levels and further increases lysine content, indicating the compensatory mechanism of protein accumulation in the seed (Wu and Messing, 2011, 2012). Still, even the dominant double RNAi against α-zeins is recessive to the QTLs of QPM (described earlier), consistent with their function in *o2* mutants. Breeding, however, is hampered by recessive traits. First, the recessiveness of *o2* can be overcome with RNAi, but the recessiveness of RNAi to the QTLs of QPM requires an additional linked neutral marker such as GFP to follow the inheritance of the RNAi locus. Presence of the QPM QTLs can produce green kernels containing the double RNAi against α-zein and high-lysine content, providing a new introduction strategy for QPM into diverse genetic backgrounds (Wu and Messing, 2011).

The same strategy applies to the long-term selection of high protein content, which can be 2.5 times higher than in normal lines (Moose *et al.*, 2004). Because this selection has primarily increased α-zeins, it actually has worsened protein quality. Using the double RNAi described earlier, however, α-zeins can be reduced to lower levels, whereas total protein levels remain unchanged owing to increased levels of nonzein proteins, probably secondary to post-transcriptional regulation of protein synthesis. The remaining levels of α zeins are sufficient to prevent an opaque kernel phenotype, and the increased nonzein proteins result in a balanced amino acid composition, combining three long-sought traits in cereals, high-lysine, high-protein, and hard seed (Wu and Messing, 2012).

Another zein locus that is differentially regulated at the post-transcriptional level is the δ-zein locus, which is regulated by the δ-zein regulator (*dzr1*). The regulator appears to target the UTRs of the 10-kDa δ-zein because a transgene with substituted UTRs became insensitive to *drz1* regulation (Lai and Messing, 2002). Certain inbred lines harbor a *dzr1* allele that is subject to genomic imprinting (Chaudhuri and Messing, 1994). There are also inbred lines, where δ-zeins are knocked out either by frame shift or transposon insertion, indicating that expression of δ-zein may not be essential (Wu *et al.*, 2009). Such natural knockouts have not been found for any other zein loci.

When δ-zeins are overexpressed (Lai and Messing, 2002), it occurs at the expense of γ-zeins and β-zeins (Wu *et al.*, 2012). Reciprocally, if γ-zeins and β-zeins are reduced with RNAi, δ-zeins increase. The connection here is the reduced sulfur pathway in plants. The γ-zeins are high in cysteine, which donates its sulfur moiety to methionine. So if cysteine is low because of increased γ-zein levels, less methionine is synthesized, starving δ-zein protein synthesis (Wu *et al.*, 2012).

With respect to protein body formation, the 10-kDa and 18-kDa δ-zeins colocalize with the α-zein protein core, but a specific role is unknown. When the above-described double δ-zein null mutant was crossed into the individual γ-zein and β-zein RNAi lines, the mild effects of these RNAi constructs on protein body formation were not exacerbated (Wu and Messing, 2010a). The lack of an obvious defect resulting from δ-zein elimination may reflect their naturally low abundance and,

perhaps, small contribution to protein body architecture. Alternatively, it may indicate that δ-zeins have evolved simply as a store of methionine and are not essential for sequestration of other zeins. However, because the effects of the δ-zein null on the combined γ-zein and β-zein RNAi line or the triple z1C/γ-/β-RNAi line were not investigated (Wu and Messing, 2010a), a structural role for the δ-zeins cannot be ruled out.

For many years, it was believed that there is a common transcriptional activator for all zein genes, and that could be the PBF described earlier (Vicente-Carbajosa *et al.*, 1997; Wang *et al.*, 1998; Jaenicke-Despres *et al.*, 2003). Because no mutant collection has yielded a knockout mutation, it was assumed that such a mutation would produce defective kernels. RNAi against PBF could provide a useful alternative approach because it is only a reduction in gene expression and not a knockout mutation. Such an RNAi line reduces α-zein and γ-zein expression only and not the other ones (Wu and Messing, 2012). It appears that the promoters of the zein superfamily have diverged rapidly because they differ also in their response to O2 as described earlier. A combination of the PBF RNAi and the *o2* mutation gives an additive effect in the reduction of zein gene expression. The same study shows that constitutively expressed PBF and O2 can transactivate transgenic zein promoters in leaf tissue but not endogenous ones. Methylation analysis of the promoters indicates that they also differ in their epigenetic marks. Unexpectedly, one of them, the β-zein promoter, could be turned on in leaf tissue in the absence of RDR2 caused by the *mob1* mutation, which controls the siRNA pathway (Wu and Messing, 2012). These lines of experimentation with different RNAi constructs illustrate the evolution of the seed as sink for reduced nitrogen and sulfur, regulated at the post-transcriptional and transcriptional level.

Conclusion

After a hiatus of research activities in the field of storage proteins compared with the beginnings of plant biochemistry and molecular biology, progress in deeper insights of their evolution and function has gained momentum in recent years because of new methods in genomics, cell biology, and RNA interference. Unexpectedly, studies of their expression during seed development has given us plethora of mechanisms of the cell machinery in general because their accumulation uses more factors than previously believed. Therefore, these studies provide a better understanding of seed development and serve as the platform for a new green revolution.

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9 Improving Grain Quality: Wheat

Peter R. Shewry

Introduction

The annual world production of cereals during the period 2007–2009 was almost 2500 million tonnes, of which about 27% was wheat (a mean of >660 million tonnes) (http://faostat.fao.org/). In terms of total production, wheat is the third most important cereal crop in the world, after maize and rice (812 million and 677 million tonnes pa during 2007–2009). However, wheat is grown over a much wider geographical and climatic range than rice and maize, from 67° N in Scandinavia and Russia to 45° S in Argentina, including higher elevations in the tropics (Feldman *et al.*, 1995). Wheat is the staple food in much of the temperate world, including North America and Europe, as well as the Middle East and North Africa. Demand for wheat products is also increasing in regions of Asia and sub-Saharan Africa, which are climatically unsuited for wheat production.

Wheat has three main end uses – human food, livestock feed, and industrial uses (notably brewing, distilling, and biofuel production) – which vary in relative importance in different countries and differ in their quality requirements. However, before considering these, it is necessary first to discuss the structure and composition of the grain.

Grain Structure and Composition

The mature wheat grain comprises three parts: endosperm, embryo, and outer layers (Figure 9.1). The endosperm accounts for ∼90% of the dry weight of the mature grain, with the outer aleurone layer (consisting of a single layer of cells in wheat) accounting for ∼6.5% and the starchy endosperm accounting for ∼83% of the grain dry weight (Table 9.1) (Barron *et al.*, 2007). The aleurone cells have thick walls, which account for about half of their dry weight and high contents of minerals bound to phytate (∼15% of the aleurone dry weight) (Hemery *et al.*, 2009), protein (∼23% dry weight) (Jensen and Martens, 1983), and lipids (4.6%–8.9% dry weight) (Chung *et al.*, 2009) but little or no starch. By contrast, the starchy endosperm accounts for ∼83% of the grain dry weight and is rich in starch (∼78% of the tissue dry weight) (Hemery *et al.*, 2009) and protein (which varies in amount with nutrition but is generally about 10% dry weight). However, the starchy endosperm is not a homogeneous tissue but comprises several cell types with gradients in the content and composition of the major components; this is illustrated for protein, starch, and cell wall polysaccharides in Figure 9.2. In particular, the outer two or three layers of starchy endosperm cells (called

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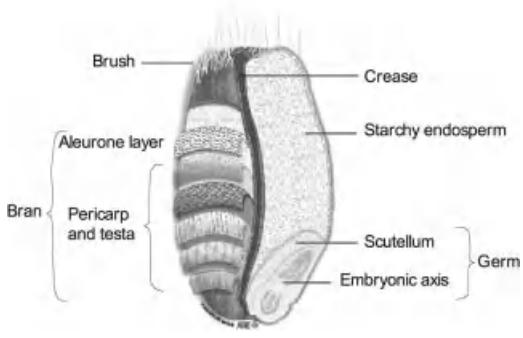

Figure 9.1 Component tissues of wheat grain. (From Surget and Barron [2005], with permission.) (For color detail, see color plate section.)

sub-aleurone cells) are rich in protein, which can account for >50% of the dry weight of flours derived from these cells (Kent, 1966), and contain little starch.

The embryo accounts for ~3% of the grain dry weight (Table 9.1) and resembles the aleurone in being rich in lipid and protein, whereas the outer layers (consisting of the outer and inner pericarp, testa, and nucellar epidermis [also called hyaline layer]) comprise 7%–8% of the grain dry weight (Table 9.1) and are rich in cell wall polysaccharides: up to 70% of the nucellar epidermis, 50% of the outer pericarp, and 44% of the testa + inner pericarp + nucellar epidermis (Barron *et al.*, 2007). The embryo, aleurone, and, to a lesser extent, the outer layers are enriched in many components that are known or considered to have health benefits for humans, including dietary fiber, minerals, vitamins, and phytochemicals. This has implications for grain processing and for the development of wheats with enhanced health benefits. The literature on these "bioactive" components is vast; Piironen *et al.* (2009) have provided an excellent account of the distributions of micronutrients and phytochemicals in the wheat grain, and Saulnier *et al.* (2007) and Stone and Morell (2009) have reviewed the distribution and properties of cell wall polysaccharides, which are the major components of wheat dietary fiber.

Most of the wheat used for human consumption is dry milled, using a complex series of rolling and sieving processes that are designed to separate the white flour derived from the starchy endosperm from the bran that contains the other botanical components. The germ (embryo) is usually present in

Table 9.1 Histological Composition of Wheat Mature Grains from Two Wheat Cultivars (Caphorn and Crousty) (% Dry Weight)

	Caphorn	Crousty
Embryo	3.0	3.2
Embryonic axis	1.5	1.7
Scutellum	1.5	1.5
Endosperm	89.2	90.1
Starchy endosperm	82.7	83.7
Aleurone layer	6.5	6.4
Outer layers	7.8	6.7
Intermediate layer[a]	3.8	3.2
Outer pericarp	4.0	3.5

[a]Composed of nucellar epidermis (hyaline layer), testa, and inner pericarp.
From Barron *et al.* (2007), with permission.

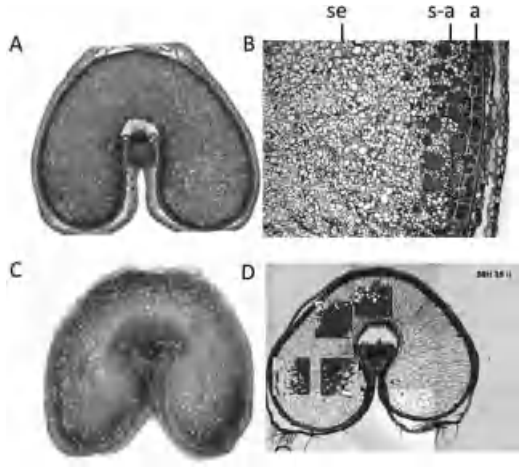

Figure 9.2 Transverse sections of developing caryopses of wheat, showing gradients in composition in the endosperm. *A* and *B,* Low-magnification and high-magnification images of a transverse section of the whole caryopsis at about 28 days after anthesis, stained with toluidine blue to show protein. Aleurone *(a)* and subaleurone *(s-a)* cells are rich in protein, and central starchy endosperm *(se)* cells are rich in starch granules (unstained). *C,* Expression of low-molecular-weight glutein subunit-promoter-GUS fusion construct in a developing endosperm, showing high expression in the subaleurone and outer starchy endosperm cells. *D,* Overlaying of Fourier-transform infra-red microspectroscopy images onto a section of cell walls only at 35 days after anthesis. The images are colored to show the distributions of high-substitution (shown in blue) and low-substitution (shown in green) forms of arabinoxylan in the cell wall. (*A* and *B,* Courtesy Cristina Sanchez-Gritsch and Paola Tosi, Rothamsted Research; *C,* courtesy Caroline Sparks and Huw Jones, Rothamsted Research; *D,* from Toole *et al.* [2010] with permission.) (For color detail, see color plate section.)

the bran fraction but can be recovered separately using specialized milling procedures. The recovery of white flour (the "extraction rate") is usually ~75% of the grain weight. Increasing this rate results in increasing proportions of the bran fraction in the flour, with "brown" flours having extraction rates of ~85% and wholemeal flours having extraction rates of 100%. In practice, these high extraction flours are usually produced by blending flour and bran fractions produced by milling, although the outer layers can also be removed before milling by friction (peeling) or abrasion (pearling), facilitating the recovery of clean aleurone-enriched fractions.

End Use Quality

The grain quality requirements are determined by the end use, which in this chapter are considered under four headings: food processing, human health, livestock feed, and fermentation for fuel and beverages. Table 9.2 provides a summary of the requirements for different end uses.

Food Processing

Wheat is consumed by humans only after extensive preparation. The most widely consumed products are bread and noodles, which are produced from hexaploid bread wheat (*Triticum aestivum*), and pasta, which is produced from tetraploid durum wheat (*T. turgidum* var *durum*). For these products, the grain is milled and the flour is mixed with water to form dough, which is either baked into bread, with or without prior fermentation, or formed into pasta or noodles. The major quality criterion for

Table 9.2 Wheat Quality Requirements

	Usage (Million Tons per Year)	Starch	Protein		Cell Wall Fiber	Texture (Milling)	Other
			Total	Gluten			
Breadmaking	5.6[a]	–	High	Strong	Low	Hard	
Biscuits and cakes		–	Medium	Weak	Low	Soft	
Human health		High amylose	–	–	High	–	High micronutrients
Distilling	0.7	High	Low	Weak	Low	Soft	
Biofuel	1.5–2.0	High	Low	Weak	Low	Soft	
Livestock feed	6	High	Low	Weak	Low	Soft	Protein quality Low phytate

[a]Breadmaking and biscuits and cakes combined.

these end uses is dough strength, which is determined largely by the amount and composition of the gluten proteins.

The gluten proteins form a network in dough that provides cohesive properties and, in the case of leavened bread, allows the entrapment of carbon dioxide released during fermentation (proofing). This entrapment expands the dough to give a porous structure, which is fixed during baking to give an acceptable crumb structure to the finished loaf. Although durum wheat grains are inevitably hard in texture, bread wheat lines differ in texture, and hard wheats are preferred for breadmaking because the strong adhesion between the starch granules and gluten proteins results in starch damage during milling, allowing water absorption during breadmaking. Lower protein contents, weaker dough strength, and soft texture are preferred for making cakes and biscuits.

Although cell wall arabinoxylan (AX) accounts only for ∼1.3%–2.7% of the dry weight of white flour (Gebruers *et al.*, 2008), it is known to have both positive and negative effects on breadmaking performance (Courtin and Delcour, 2002). Bakers often use xylanases during breadmaking to optimize the structure, solubility, and viscosity of the flour AX.

Human Health

Both short-term dietary interventions and longer term epidemiological studies have shown that whole-grain foods are able to provide protection against the risk of many chronic diseases, particularly the combination of conditions known as the metabolic syndrome (which include type 2 diabetes, obesity, and cardiovascular disease (Krauss *et al.*, 2000; Nugent, 2004; de Munter *et al.*, 2007; Mellen *et al.*, 2008)). Although this protection may result from a combination of beneficial components, including phytochemicals, the evidence for the effects of dietary fiber and for the importance of both soluble and insoluble forms is particularly compelling (Slavin, 2004; Lunn and Buttriss, 2007; Topping, 2007; Wood, 2007; Buttress and Stokes, 2008; Anderson *et al.*, 2009; Fardet, 2010). It has also been suggested that the slow release in the colon of ferulic acid bound to dietary fiber has health benefits (Vitaglione *et al.*, 2008).

Because wheat products are major sources of dietary fiber in many diets (e.g., cereals and cereal products account for >40% of the daily intake in adults in the United Kingdom (Buttriss and Stokes, 2008) and bread alone for 20% (Steer *et al.*, 2008)), the improvement of the dietary fiber content and composition of wheat flour is an attractive approach to improve the health of large populations of consumers with modest cost. Reducing the glycemic index of foods is important in relation to decreasing the risk of type 2 diabetes, and high soluble dietary fiber may contribute to this by increasing the intestinal viscosity and decreasing the rate of absorption of sugars. An alternative,

and complementary, approach to reducing the glycemic index is to develop wheats with resistant starch. Resistant starch has been classified into four types, with high amylose starch contributing to types RS2 (resistant to digestion) and RS3 (retrograded starch).

Livestock Feed

Wheat is widely used for livestock feed, particularly in Europe, where it is combined with protein-rich feeds from legumes or oilseeds. In many countries, feed wheat is considered primarily as a source of starch (calories), and the emphasis in breeding feed varieties is on high yield (i.e., high starch) and low protein.

The quality of the grain for livestock feed is also affected by the fiber content, with high levels of dietary fiber components, and particularly of soluble fiber, being detrimental to feed quality owing to high viscosity. This is a particular problem for chickens and other poultry in which it leads to sticky feces, and feed processors may use endoxylanase and β-glucanase enzymes to reduce the viscosity of the feed (Pettersson and Åman, 1989). However, this is an expensive option compared with developing low-fiber feed grain. Distillers dried grains and solubles (DDGS) from distilling and biofuel production (see subsequently) are also enriched in fiber (>30% dry weight), which may result in high viscosity and require enzyme treatment when used for livestock feed.

Distilling and Biofuel Production

Substantial volumes of wheat are used for alcohol production, either for distilling for beverages or for bioethanol production. A low content of dietary fiber is desirable for these processes, not only to increase alcohol yield (because low dietary fiber grain contains a higher proportion of starch) but also to reduce the viscosity, which results in technical problems during processing. A high fiber content also reduces the quality of the DDGS for feed, as discussed previously.

Redesigning the Grain

It is impossible to develop a single type of wheat suitable for all end uses. Consequently, most wheat breeders in the United Kingdom and other European countries maintain two or more parallel breeding programs: for high-yielding, low-protein types for livestock feed and for distilling and bioethanol production; for high-protein types with strong gluten and hard texture for breadmaking; and, in some cases, for soft wheats with weak gluten for biscuits and cakes. Although most research has focused on single components and end uses, it is now possible to take a more integrated approach, ascertaining the processes and mechanisms that determine the development and structure and composition of the mature grain. These should ultimately allow a more rational approach to be taken, in which the grain can be specifically designed for different end uses.

Manipulation of Grain Protein Content and Quality

Grain Protein Content

Selection for protein content can result in massive differences between cereal lines, the classic illustration being the long-term Illinois experiment in which 70 generations of selection resulted

in maize lines with protein contents ranging from 4.4%–26.6% (see Chapter 13) (Dudley *et al.*, 1974). More modest differences result when selection is carried out as part of a conventional breeding program; breadmaking and feed wheats in the United Kingdom differ in protein content by ~2% dry weight when grown under similar conditions (Snape *et al.*, 1993). Breeders have also exploited genetic sources of high protein in wild species related to wheat, including an *Aegilops* species, which donated a high-protein gene to the Kansan variety Plainsman V (Finney, 1978), and, in particular, wild lines of emmer (*Triticum turgidum* var *dicoccoides*), a tetraploid species related to cultivated emmer and durum wheats (Avivi, 1978). The latter is the source of the *Gpc-B1* gene, which has been transferred into commercial wheats (Khan *et al.*, 1989, 2000; Mesfin *et al.*, 2000). The *Gpc-B1* gene encodes a transcription factor that accelerates senescence of the vegetative tissue, increasing the mobilization and transfer of nitrogen and minerals (iron and zinc) into the grain (Distelfeld *et al.*, 2006; Uauy *et al.*, 2006). However, the impact of *Gpc-B1* on grain yield is still not established, with negative (Chee *et al.*, 2001) and neutral (Mesfin *et al.*, 2000) effects being reported. Although *Gpc-B1* accounts for 70% of the variation in grain protein content in crosses (Chee *et al.*, 2001; Joppa *et al.*, 1997), no other major loci have been discovered with QTL being reported on numerous chromosomes. Worland and Snape (2001) suggested that <30% of the variation in grain protein content in winter wheats in the United Kingdom could be explained by known genes.

Grain Protein Quality

The content of essential amino acids is relevant to the use of wheat for livestock and for humans where wheat forms a major part of the diet, with wheat resembling other cereals in being particularly deficient in lysine (see Chapter 8) (Shewry, 2007). However, the main target for improving wheat protein quality is for breadmaking, which is largely determined by the gluten proteins.

Gluten Proteins

Wheat gluten can be defined as the proteinaceous mass that remains when dough is washed to remove soluble components, starch, and other cellular material. It comprises about 80% protein, 10% starch (which is probably entrapped within the protein), and 10% other material (e.g., lipids, minerals, polysaccharides) on a dry weight basis, with most of the protein components being prolamin storage proteins from the starchy endosperm cells. These proteins come together when wheat flour is mixed with water, forming a continuous network in the dough. This network provides the unique viscoelastic properties that enable the dough to be processed in bread, other baked products, pasta, and noodles. The quality of wheat for breadmaking is often limited by low elasticity (strength), particularly wheat grown in cooler temperate climates, resulting in poor gas retention during proofing, low loaf volume, and dense crumb structure.

The gluten proteins are classified into two broad groups, called gliadins and glutenins, which are present in about equal amounts. These fractions were initially defined on the basis of their solubility (gliadins) or insolubility (glutenins) in aqueous alcohols (e.g., 70% ethanol), which result from their presence as monomers and polymers. The polymeric glutenin proteins have been studied in the most detail because dough strength is positively related to the amount and size of the high-molecular-weight (HMW) glutenin polymers (Wieser *et al.*, 2006).

Glutenins and Dough Strength

The glutenin polymers are stabilized by interchain disulfide bonds, which can be reduced to release groups of HMW and low-molecular-weight subunits. Although the HMW subunits are the least abundant group of gluten proteins, accounting for ~10% of total gluten (see subsequently) and ~20% of glutenin, they have been studied in the greatest detail because of their role in determining gluten and dough elasticity. This role is demonstrated by two lines of evidence. First, the HMW subunits are concentrated in HMW glutenin polymers, which are known to contribute to dough strength (Huebner and Wall, 1976; Field *et al.*, 1983). Second, genetic studies have shown that allelic variation in HMW subunit composition is correlated with differences in dough strength and breadmaking quality (Burnouf and Bouriquet, 1980; Moonen *et al.*, 1982; Cressey *et al.*, 1987; Lawrence *et al.*, 1987; Payne, 1987).

HMW Subunits of Glutenin

Cultivars of bread wheat contain three, four, or five HMW subunits of glutenin. These are encoded by the *Glu-1* loci on the long arms of the group 1 chromosomes, with each locus comprising two genes, which encode an x-type and a y-type subunit. The x-type and y-type proteins were initially defined by their mobility on SDS-PAGE (y-type being faster) (Payne *et al.*, 1981) but are now known also to differ in their amino acid sequences, including numbers of cysteine residues available for polymer formation (Shewry *et al.*, 2003). The genes encoding x-type and y-type subunits are tightly linked, and recombination occurs only rarely.

The variation in HMW subunit number in bread wheat results from gene silencing, with 1Ay subunits never being expressed and 1Ax and 1By subunits being expressed in some lines only. However, 1Ay subunits may be present in wild-type diploid and tetraploid wheats (Waines and Payne, 1987; Levy *et al.*, 1988; Margiotta *et al.*, 1998), and the number of HMW subunits expressed in bread wheat could be increased by introgression of 1Ay subunits from these species.

As mentioned earlier, genetic variation in HMW subunit number and composition is correlated with differences in dough strength and breadmaking performance, and this probably results from both quantitative and qualitative effects. First, the expression of a 1Ax subunit is associated with good quality compared with the null (silent) form, as is the expression of subunit 1Bx7 in combination with subunit 1By8 or subunit 1By9, rather than subunit 1Bx7 alone (Payne *et al.*, 1987). It has been shown that the individual subunit proteins each account, on average, for ~2% of the total grain protein (Seilmeier *et al.*, 1991; Halford *et al.*, 1992), so these effects of subunit number may have a quantitative basis, with a higher number of expressed genes resulting in more HMW subunit protein and greater dough strength.

However, other effects of HMW subunits on dough strength appear to be qualitative, resulting from allelic differences in HMW subunit structure. This has been studied in most detail for the HMW subunit pairs encoded by the *Glu-D1* locus. Two "allelic pairs" of HMW subunits encoded by chromosome 1D occur widely in commercially grown bread wheat varieties, called 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12. The 1Dx 5 + 1Dy10 allelic pair is associated with superior quality, and this effect appears to result from the presence of a higher proportion of HMW glutenin polymers, rather than more total glutenin polymers or total protein (Gupta and MacRitchie, 1994). The effect on HMW polymers may relate to the fact that subunit 1Dx5 contains an additional cysteine residue compared with subunit 1Dx2 (Anderson *et al.*, 1989), which could lead to an increase in the number

of interchain disulfide bonds that are formed and the formation of larger or more highly cross-linked glutenin polymers.

Increasing Dough Strength by Transgenesis

The availability of genes encoding HMW subunits and the demonstration of a relationship between the number of expressed genes and dough strength (discussed earlier) have resulted in numerous reports of the expression of HMW transgenes in wheat, including some of the very first reports of wheat transformation (Altpeter *et al.*, 1996; Blechl and Anderson, 1996; Barro *et al.*, 1997; Alvarez *et al.*, 2000). This work has been reviewed extensively, including two detailed more recent accounts (Blechl and Jones, 2009; Jones *et al.*, 2009), and is only briefly summarized here.

All studies have used endogenous HMW subunit promoters, and expression levels tend to be similar to or greater than those of the endogenous HMW subunit genes. However, this may be misleading because workers tend to discard lines showing low levels of transgene expression.

The expression of a transgene encoding subunit 1Ax1 can result in increased dough strength (Barro *et al.*, 1997; Vasil *et al.*, 2001), although the effect is more limited in commercial cultivars with high intrinsic dough strength (Alvarez *et al.*, 2001; Field *et al.*, 2008; Rakszegi *et al.*, 2008). High-level expression of the transgene may also lead to an overstrong phenotype (Rakszegi *et al.*, 2008).

Subunits 1Dx5 and 1Dy10 are always present as a pair, and the expression of a subunit 1Dx5 transgene on its own usually results in overstrong properties (Rooke *et al.*, 1999; Alvarez *et al.*, 2001; Rakszegi *et al.*, 2005; Blechl *et al.*, 2007), which appear to relate to an increase in glutenin cross-linking (Popineau *et al.*, 2001). This overstrong effect can be ameliorated by the incorporation of purified subunit 1Dy10 into the dough (Butow *et al.*, 2003) or by coexpression of the 1Dx5 and 1Dy10 transgenes (Blechl *et al.*, 2007). Expression of the 1Dy10 transgene on its own may also result in increased dough strength, similar to the effect observed with the subunit 1Ax1 transgene (Blechl *et al.*, 2007; León *et al.*, 2009). By contrast, Graybosch *et al.* (2011) showed that field grown lines expressing the subunit 1Dy10 transgene tended to show overstrong characteristics, whereas lines expressing 1Dx5 and 1Dy10 tended to lack well-defined peaks in Mixograph analysis.

León *et al.* (2010) compared lines expressing various combinations of the 1DAx1, 1Dx5, and 1Dy10 transgenes. They showed that the combination of the 1Dx5 and 1Dy10 transgenes gave better dough properties than the combinations of the 1Dx5 and 1Ax1 or 1Ax1 and 1Dy10 transgenes. The combination of the subunits 1Dx5 + 1Dy10 transgenes also gave greater strength than the expression of all three transgenes.

These studies demonstrate that transgenic expression of HMW subunit genes can be used to increase the dough strength of wheat, although the precise effect varies with the selection of subunits, the expression level, and the genetic background (including the intrinsic dough strength and composition of endogenous HMW subunits).

The expression levels of the HMW subunit transgenes appear to be stable over multiple generations (Barro *et al.*, 2002; Rakszegi *et al.*, 2005; Shewry *et al.*, 2006; Rakszegi *et al.*, 2008), whereas the limited number of field trials that have been carried out show that they differ little from control lines in agronomic performance (Barro *et al.*, 2002; Bregitzer *et al.*, 2006; Shewry *et al.*, 2006; Graybosch *et al.*, 2011). Finally, analyses of metabolite profiles (Baker *et al.*, 2006) and gene expression patterns (Baudo *et al.*, 2006) show that they are "substantially equivalent" to nontransgenic wheat and should be acceptable to regulatory authorities.

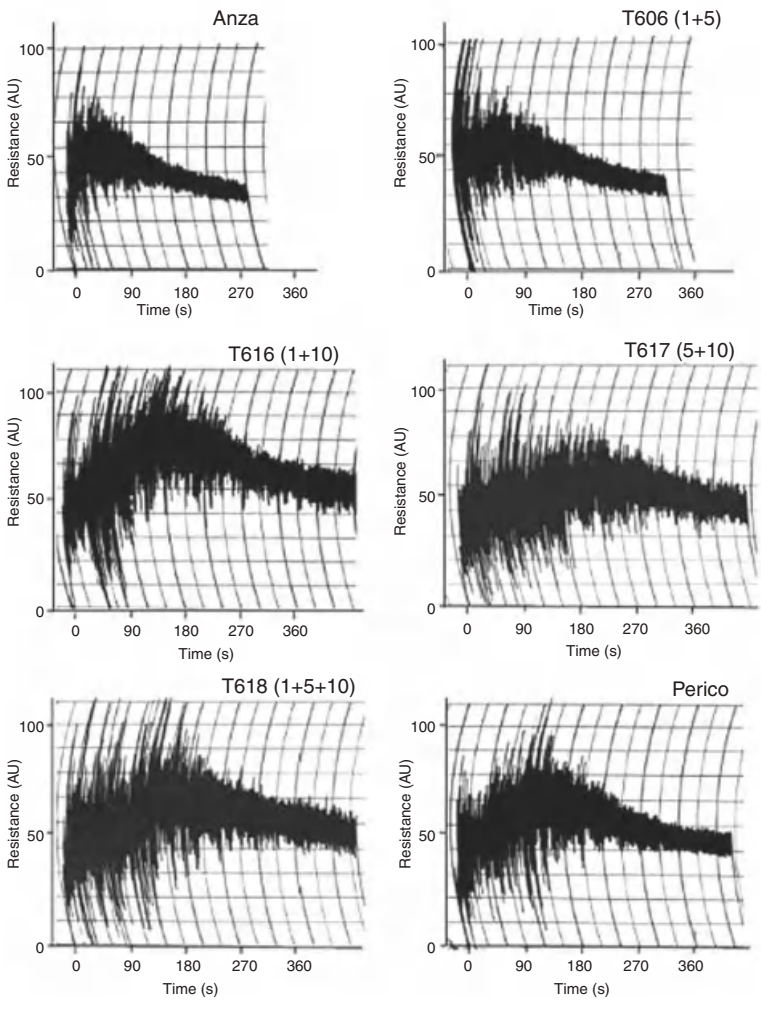

Figure 9.3 Comparison of the Mixograph curves of transgenic lines expressing additional HMW subunits (indicated in parentheses) with the control line Anza and a commercial wheat cultivar Perico. The Mixograph records the increase in stress as dough is mixed to its maximum resistance and the subsequent decrease in stress on overmixing. Numerous measurements are taken that relate to dough strength with mixing time (measured in seconds) being positively correlated with dough strength. In the experiment shown, the mixing time was 35 seconds for Anza; 128 seconds for Perico; and 63 seconds, 133 seconds, 185 seconds, and 156 seconds for transgenic lines T606 (1Ax1 + 1Dx5 transgenes), T616 (1Ax1 + 1Dy10 transgenes), T167 (1Dx5 + 1Dy10 transgenes), and T618 (1Ax1 + 1Dx5 + 1Dy10 transgenes), demonstrating that the transgenic lines compare favorably with the commercial control, with T617 having the greatest strength. (From León *et al.* [2010] with permission.)

Manipulation of Grain Texture

Kernel texture has been described as "the most important single characteristic that affects the functionality of a common wheat" (Pomeranz and Williams, 1990), determining how the grain behaves during milling and the suitability of the flour for various end uses. Between ~60% and

80% of the variation in hardness in bread wheat in controlled by allelic variation at the *Hardness* (*Ha*) locus, which is located on chromosome 5D of bread wheat, with minor hardness loci having been mapped onto numerous other chromosomes (Sourdille *et al.*, 1996; Galande *et al.*, 2001; Turner *et al.*, 2004; Weightman *et al.*, 2008). The *Ha* locus contains two genes encoding small (~15,000 kDa) sulfur-rich proteins, which are characterized by the presence of short tryptophan-rich motifs and have been named puroindoline (Pin) a and b (Douliez *et al.*, 2000). These proteins are present in greater amounts on the surface of starch granules prepared from mature grain of soft wheats than from hard wheats, and it has been suggested that they determine softness by preventing adhesion between the starch granule surface and the gluten protein matrix (Greenwell and Schofield, 1986). Consequently, the starch granules are more easily released from soft wheat during milling, requiring less energy input and with little damage. The equivalent regions of chromosomes 5A and 5B of both hexaploid bread and tetraploid wheats have independent deletions of the genes encoding puroindolines (Chantret *et al.*, 2005) and cultivated durum wheat, and other tetraploid wheats are always hard.

Most hard genotypes of bread wheat have either a deletion of the Pin a gene (*Pina-D1*) or one of six point mutations in the Pin b gene (*PinB-D1*) leading to an amino acid substitution in the encoded protein (Morris, 2002), with other types of mutations occurring more rarely (Bhave and Morris, 2008; Morris and Bhave, 2008). The point mutations are proposed to affect the binding of the puroindoline to the starch, but despite a massive volume of research, the mechanism of binding of Pins to starch granules and the impact of mutations on this remain poorly understood.

Direct effects of Pin a and b on grain softness have been demonstrated by transgenic expression in rice, a species that lacks Pins and has hard grain (Krishnamurthy and Giroux, 2001), and in hard bread wheat (Beecher *et al.*, 2002; Hogg *et al.*, 2004; Martin *et al.*, 2008). Studies have shown the existence of at least four minor variant forms of Pins encoded by loci on the long arms of chromosomes 7A, 7B, and 7D, with the 7A component mapping close to a QTL for hardness (Wilkinson *et al.*, 2008; Chen *et al.*, 2010). Other minor hardness QTL could relate to aspects of grain structure such as cell wall composition and mechanical properties.

Development of Wheat with Resistant Starch

As described in detail in Chapter 7, starch is a mixture of two glucose polymers: amylose, which comprises single unbranched (1→4) α-linked chains of up to several thousand glucose units, and amylopectin, which is highly branched (with (1→6) α-linkages and (1→4) α-linkages) and may comprise >100,000 glucose unit residues. In most species, including wheat, amylose and amylopectin occur in a ratio of 1:3 amylose to amylopectin, with only limited variation in their proportions between genotypes or in relation to the environment. High amylose starches are attractive for developing healthy foods because they are more slowly digested and become resistant on cooking, both of which lead to reduced glycemic index in the human gastrointestinal tract.

Amylose and amylopectin are both synthesized from ADP glucose, but the pathways differ. This allows the adoption of two strategies for producing high amylose starch, either increasing amylose synthesis by transgenic overexpression or reducing amylopectin synthesis by transgenesis or mutagenesis.

The synthesis of amylose in the wheat starchy endosperm requires only a single enzyme, granule bound starch synthase form Ia (BGSSIa) (Stone and Morell, 2009). In theory, it should be possible to increase amylose content substantially by a single transgenic event. However, this approach has not been reported, and work so far has focused on reducing amylopectin synthesis.

The *Starch granule protein-1* (*Sgp-1*) genes located on chromosomes 7AS, 7BS, and 7DS of bread wheat (Chao *et al.*, 1989; Yamamori and Endo, 1996) encode forms of the starch synthase II (SSII) enzyme, which are involved in the synthesis of medium-length chains in amylopectin (Stone and Morell, 1999). The encoded proteins (SGP-A1, SGP-B1 and SGP-D1) are readily identified by SDS-PAGE of proteins extracted from purified starch granules, facilitating the identification of null mutant forms and the stacking of mutations at the three loci to result in a high amylose phenotype in hexaploid bread wheat. A modest but significant increase in the proportion of amylose, from ~29% in the Chinese Spring control lines to ~37%, resulted (Yamamori *et al.*, 2000). However, the total starch content was also reduced, from 60.7% in the control Chinese Spring wheat to 49.4% in the triple mutant. The structure of the starch was also affected, with an increase in the proportion of short chains (degree of polymerization [DP] 6–10) and a decrease in longer chains (DP 11–25).

Hung *et al.* (2005) demonstrated that the incorporation of 10%, 30%, and 50% high amylose (35%) flour into bread resulted in increases in the content of resistant starch, from 0.9% dry weight in the control bread to 1.65%, 2.6%, and 3.0%. The availability of genomic sequences of the *Sgp-1* genes has also allowed the use of TILLING (targeting induced mutations in genomes (Slade *et al.*, 2005)) to identify further allelic mutations in the *Sgp-1* genes in a mutagenized wheat population (Sestili *et al.*, 2010).

A much greater impact on the amylose content of wheat starch was achieved by transgenesis, using RNAi suppression to reduce simultaneously the activities of starch branching enzymes (SBE) II (SBEIIa and SBEIIb) that are responsible for synthesizing (1→6) linkages in amylopectin (Stone and Morell, 2009). The resulting line had 74.4% amylose compared with 25.5% in the untransformed control with slightly reduced total starch content (43.4% compared with 52%) (Regina *et al.*, 2006). By contrast to the *Gsp-1* lines discussed previously, the debranched starch had a decreased proportion of short chains (DP 4–12) and an increased proportion of longer chains of DP >12. Regina *et al.* (2006) also reported that several indexes of colon health (digesta wet weight, short-chain fatty acid pools, and fecal short-chain fatty acid excretion) were increased by twofold in rats fed the wholemeal transgenic wheat compared with the control.

The practical application of high amylose wheats in food production for human consumption has been limited by the requirement to incorporate the phenotype into lines that have commercially acceptable yields and are acceptable to consumers. At the present time, neither the *Gsp-1* mutants nor the SBEII transgenics fulfill these requirements.

Improving Content and Composition of Dietary Fiber

Resistant starch and fructans may be classified as dietary fiber because "they are resistant to digestion and absorption in the human small intestine with partial or complete fermentation in the large intestine" (AACC, 2001). However, other definitions restrict the term to the nonstarch polysaccharides of plant cell walls, and this narrower definition is used here.

Wheat Grain Cell Walls

The tissues of the wheat grain differ in their content and composition of cell wall polysaccharides, as summarized in Table 9.3. The total content of nonstarch polysaccharides, including fructans, in wheat flour (derived from the starchy endosperm) is ~3.5%–4% dry weight (Knudsen, 1997; Haskå *et al.*, 2008), with the major components being cell wall polysaccharides. These comprise ~20%

Table 9.3 Compositions of Cell Wall Types in Wheat Grain Tissues (% Dry Weight)

	Cell Walls (% Dry Weight)	Cell Wall Components (% Total Polysaccharide)						
		Cellulose	Lignin	Pectin	AX[a]	β-glucan[b]	Glucomannan	Reference
Starchy endosperm	2–3	2	0	–	70	20	7	Mares and Stone, 1973; Loosveld *et al.*, 1998
Total bran (outer layers and aleurone)		29	8	–	64	6	–	Selvendran *et al.*, 1980
Aleurone	40	2–4	0	–	62–65	29–34	–	Bacic and Stone, 1981; Rhodes and Stone, 2002; Antoine *et al.*, 2003
Outer pericarp (beeswing)		30	12	–	60	–	–	Du Pont and Selvendran, 1987
Straw	85	37–40	14–17	0.5	39	–	–	Lawther *et al.*, 1995; Sun *et al.*, 2005

[a] Arabinoxylan varies in structure with highly cross-linked and substituted forms (GAX) being present in outer layers and straw.
[b] (1→3,1→4)-β-D-glucan. Traces of (1→3)-β-D-glucan (callose) may also be present in grain fractions.
Modified from Shewry *et al.* (2010).

(1→3,1→4)-β-D-glucans (β-glucan), 70% arabinoxylan (AX), 2%–4% cellulose, and 2%–7% glucomannan (Stone and Morell, 2009). Andersson *et al.* (1992) showed that the glucose content of the nonstarch polysaccharides (which was presumably derived from β-glucan) ranged from 0.25%–0.63% dry weight (mean 0.44% dry weight). More detailed analyses of AX were reported by Gebruers *et al.* (2008), who determined total AX (TOT-AX) and water-extractable AX (WE-AX) in flours of 150 wheats grown on the same site in Hungary. TOT-AX ranged from 1.35%–2.75% dry weight (mean 1.93% dry weight) and WE-AX from 0.30%–1.40% dry weight (mean 0.51% dry weight). No lignin is present in white flour, and little is known about the extent of variation in the contents of cellulose and glucomannan.

Barron *et al.* (2007) showed that the sugars released on hydrolysis of the nonstarch polysaccharides present in the isolated aleurone layer accounted for 34.0% and 42.4% of the dry weight in two cultivars, with the major sugars being arabinose, xylose, and glucose. These analyses agree with the data in Table 9.3 (which are taken from Stone and Morell, 2009) showing that the composition of the aleurone cell walls is similar to that of the starchy endosperm cells except that the proportion of AX is slightly lower and that of β-glucan slightly higher.

The outer layers of the wheat grain, comprising the nucellar epidermis, testa, and inner and outer pericarp, have been studied in less detail. However, available data indicate that the pericarp (which is the major component of the outer layers) has a similar composition to vegetative tissues, comprising mainly cellulose, lignin, and glucuronoarabinoxylans with little or no β-glucan (Table 9.3) (Barron *et al.*, 2007; Stone and Morell, 2009).

Three approaches can be taken to increase the dietary intake of fiber in wheat products. The first is to increase the consumption of whole-grain foods, or foods made from flours that contain a proportion of the aleurone fraction. Most food products are made from white flour, which corresponds to ~70%–75% of the whole grain. Increasing the flour extraction rate to ≥80% results in a browner flour with an increasing proportion of the aleurone and outer layers. The second approach is to remove and fractionate the bran and to recombine defined aleurone fractions with white flour. However, both of these approaches may adversely affect the functional properties of the flour and the appearance and

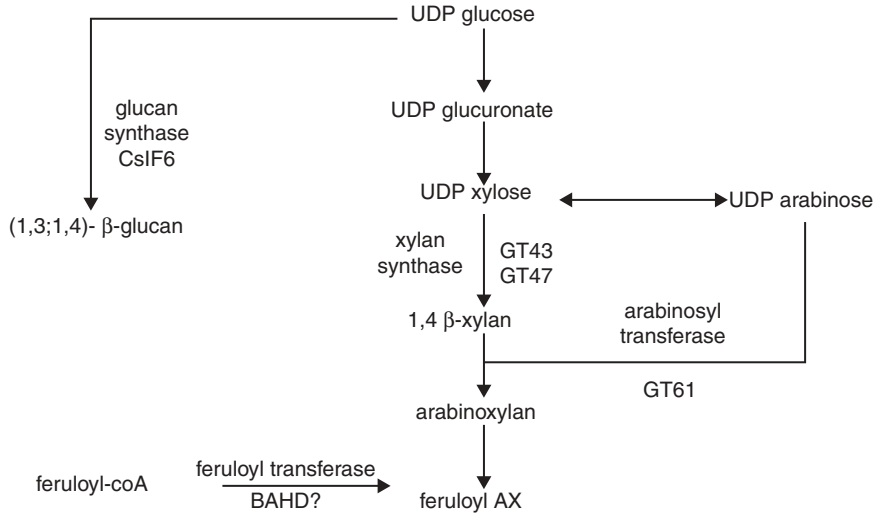

Figure 9.4 Proposed pathway of arabinoxylan synthesis. The gene families that have been proposed to encode xylan synthase, arabinosyl transferase, and feruloyl transferase are indicated.

consumer acceptability of the product and may have cost implications. It is more attractive in the long run to improve the fiber content and composition of white flour (i.e., the starchy endosperm). We have adopted the latter approach, by identifying candidate genes for key steps in β-glucan and AX synthesis (Figure 9.4) and characterizing their functions by RNAi suppression in the starchy endosperm of transgenic wheat.

Manipulating β-glucan

(1→3,1→4)-β-D-glucan (β-glucan) comprises glucose residues joined by (1→3) and (1→4) linkages (Figure 9.5*A*). Single (1→3) linkages are usually separated by two or three (1→4) linkages, but longer stretches of (1→4) linked glucan of up to 14 units have been reported for wheat bran β-glucan (Liu *et al.*, 2006). Such regions are sometimes referred to as "cellulose-like" because cellulose is (1→4)-β-D-glucan without any (1→3) linkages. The distribution of (1→3) and (1→4) linkages may determine the solubility of β-glucan and the viscosity of aqueous extracts (Lazaridou and Biliaderis, 2007).

Burton *et al.* (2006) showed that transformation of *Arabidopsis* (a species that does not normally synthesize β-glucan) with the *CSLF6* gene of rice resulted in the accumulation of β-glucan in leaves, and we have since shown that RNAi suppression of the endogenous *CSLF6* gene in developing wheat endosperm resulted in a mean decrease in total β-glucan of 42% in five transgenic lines (Nemeth *et al.*, 2010). Similarly, Burton *et al.* (2011) showed that overexpression of *CSLF6* in transgenic barley resulted in increases of >80% in the content of β-glucan in seeds.

Although these studies demonstrate that *CSLF6* encodes a β-glucan synthase, it is unclear whether this enzyme catalyzes the synthesis of both (1→3) and (1→4) linkages. However, the RNAi suppression of *CSLF6* in transgenic wheat resulted in a reduction in the molecular weight profile of the β-glucan extracted by hot water, whereas it had little effect on either the proportion of the total fraction that was extracted or the ratio of glucan fragments of DP 3 and DP 4 released by

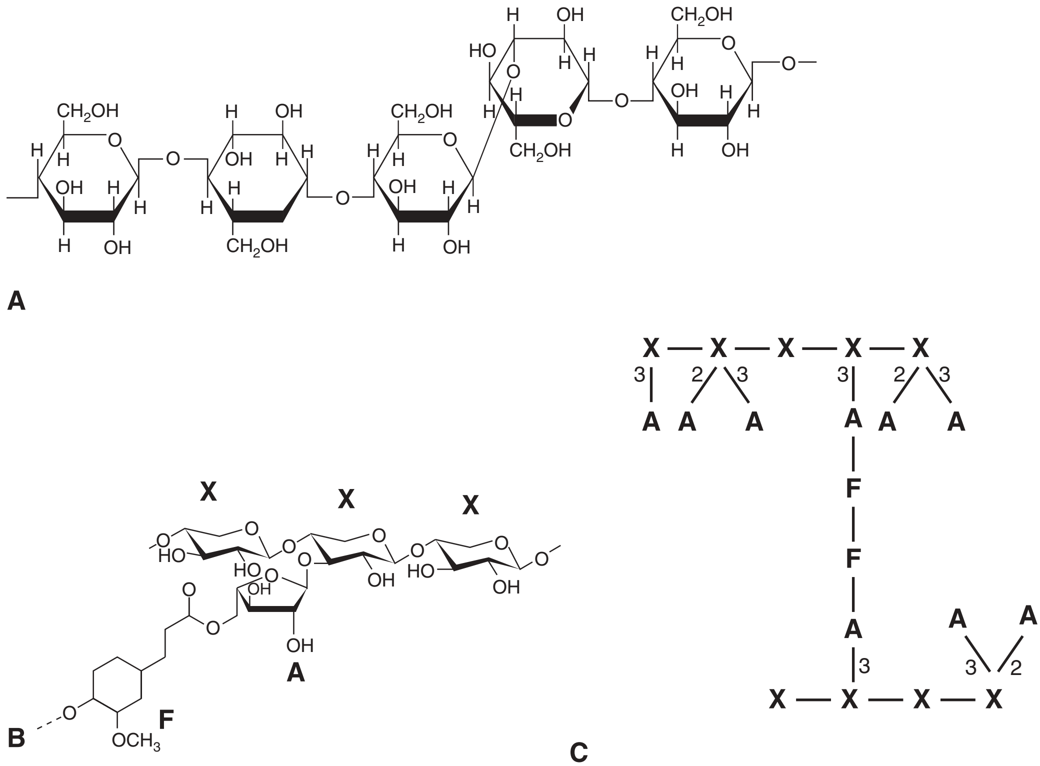

Figure 9.5 Structures of cell wall polysaccharides of wheat starchy endosperm. *A,* (1-3,1-4)-β-D-glucan. *B,* Arabinoxylan structure. *C,* Arabinoxylan schematic. A, arabinose substitution; F, ferulic acid substitution; X, (1→4) linked β-D-xylopyranosyl units.

digestion of the total β-glucan with lichenase (an *endo*-(1-3)(1-4)-β-D-glucan-4-glucanohydrolase, which was increased only slightly) (Nemeth *et al.*, 2010).

Manipulating Arabinoxylan

Arabinoxylan has a more complex structure than β-glucan, comprising a backbone of β-(1-4)-linked D-xylopyranosyl residues, which may be substituted with arabinose at the 0-3 position or at the 0-2 and 0-3 positions (Figure 9.5*B* and *C*). The 0-3-linked arabinose may also be esterified with ferulic acid or, more rarely, *p*-coumaric acid, linked to the 0-5 position. Adjacent ferulic acid groups present on AX may form diferulate (or, more rarely, triferulate) cross-links, via an oxidative process that generates a stable ether bond.

This complex structure provides a basis for considerable diversity in AX structure, which may result in differences in solubility, viscosity, and health benefits. In particular, highly arabinosylated AX may be more soluble in water owing to steric hindrance of hydrogen bonding between the xylan backbone, whereas cross-linking results in the formation of highly hydrated but insoluble gels.

Mitchell *et al.* (2007) used a bioinformatics approach to identify candidate genes for the biosynthesis and ferulylation of AX, including analysis of expression profiles in developing whole grain using

Affymetrix arrays (Toole *et al.*, 2010) and in developing starchy endosperm using high-throughput sequencing (Pellny *et al.*, 2012). This approach identified genes in the glucosyl transferase (GT) 43 and GT 47 families as encoding xylan synthases and in the GT 61 family as encoding arabinosyl transferase (Figure 9.4), and these identifications have been confirmed by RNAi suppression in transgenic wheat (Anders *et al.*, 2012; unpublished results of Wilkinson M, Lovegrove A, Freeman J, Pellny TK, Weimar T, Baker J, Shewry PR and Mitchell RAC). Similarly, genes in the acyl-CoA transferase superfamily (PF02458) were predicted to encode arabinoxylan feruloyl transferases (Figure 9.4). Piston *et al.* (2010) showed that the simultaneous downregulation of four genes in this family resulted in a modest but significant decrease in the ferulylation of cell wall AX in leaves of rice plants.

Our knowledge of dietary fiber synthesis in wheat is still incomplete, with important questions relating to the determination of the relative contents of β-glucan and AX, the linkage structure and solubility of β-glucan, and the fine structure (particularly the specificity of arabinosylation) and ferulylation of AX. Nevertheless, we should be in a position within the next few years to develop wheats with specific AX contents, compositions, and properties, exploiting a combination of natural variation, mutagenesis, and transgenesis.

Conclusion

This chapter has focused on aspects of grain quality that are determined by starch, proteins, and cell wall polysaccharides, which are the three major groups of components in the grain and together account for ≥90% of the grain dry weight. All are now well understood and amenable to manipulation by classical genetics or modern molecular approaches. However, this does not mean that they are the only quality determinants or even the most important for some applications.

Wheat is also an important source of minerals (particularly iron, zinc, and selenium), vitamin E (tocols), and B vitamins (including folates) for the human diet; a range of phytochemicals (including phenolic acids, lignans, flavonoids, and alkylresorcinols) may also have health benefits. By contrast, the accumulation of free asparagine may contribute to the formation of acrylamide (a carcinogen) during processing. Unraveling the control of grain development and composition would also facilitate the manipulation of this wider and more challenging range of targets.

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10 Legume Seed Genomics: How to Respond to the Challenges and Potential of a Key Plant Family?

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Introduction

What Is Special about Legume Seeds?

The Fabales represent the third largest flowering plant family, with members ranging from tiny forage species to impressive rainforest trees. In addition to their biodiversity, legumes are of particular importance because of their ability to fix atmospheric nitrogen by a symbiotic relationship with rhizobacteria. Many legumes also benefit from mycorrhizal symbioses that supply phosphate and other mineral ions and help regulate water homeostasis. Legume seeds have always been an important source of protein in the human diet, and current agricultural practice also relies on legumes as a source of protein for animal feed. For example, peas are used for human consumption and for animal feed, particularly for poultry and to a lesser extent pigs. Compared with soybean, peas contain less protein (22%–25% as opposed to 35%). In addition to their widespread use as a source of fixed nitrogen in crop rotations and pastures and as a source of protein, cultivated legumes have a wide range of other uses, the most important of which are as renewable fuel and biomaterial sources and for combating desertification.

The use of legume crops as a source of nitrogen is crucial for sustainable agriculture, synthetic N fertilizer production being an energy-intensive process. Despite their potential, the area sown to legumes is small in many countries and the breeding effort modest compared with cereals or other major crops. Many legume seed crops contain antinutritional components, which can compromise their utility in feed and food, and removal of these components constitutes an important breeding objective (Jezierny *et al.*, 2010). The efforts made in improving seed protein content, bioavailability, and nutritional quality of legume seeds have been summarized by Burstin *et al.* (2011). Genomics can contribute to the breeding effort by providing tools for the identification of genes encoding key traits such as seed protein content and quality and by generating markers applicable for selection in many legume crops.

Oilseed and Starch Pulses

Legumes employ different and species-specific strategies for carbon storage, some storing principally lipids, such as soybean, and others storing mainly starch, such as pea, chickpea, and faba

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bean; a third group stores carbon skeletons mainly as protein (e.g., *Medicago truncatula*). Grains of noncrop legumes generally also contain appreciable quantities of carbon in the form of cell wall carbohydrates. Legume seed feedstocks are important energy as well as protein sources, and legume breeding contrives to maximize seed calorific content in addition to its protein content. Because peas accumulate starch and not lipids, they have a lower calorific value than soybeans. In their favor, they do not require irrigation in Europe, and they have a measure of cold tolerance compared with soybean. Breeders have long known that in soybean the two major reserve substances, protein and triglyceride, are metabolically linked, and their level can be selected as a trait. A shortfall of accumulation of a major reserve substance may limit the availability of critical nutrients for the postgermination seedling. Suppression of seed storage protein production results in compensating nonstorage protein accumulation, shown by mutation-induced suppression or genetic modification of storage protein synthesis in soybean (Kinney *et al.*, 2001; Takahashi *et al.*, 2003), all resulting in rebalancing protein content by increased accumulation of other seed proteins. Seed protein composition is also strongly influenced by nutrient availability, as shown by the ability of legume seeds to compensate for low levels in the relatively poor 11S globulins under sulfur limiting conditions by increasing 7S globulins as a way to maintain seed protein content (Sexton *et al.*, 1998). The accumulation of different seed storage components (protein, lipid, and starch) is inversely linked in that increased protein content is accompanied by reduced lipid deposition and vice versa (Schmidt and Herman, 2008). Lipid and protein reserves are kept within narrow limits, presumably corresponding to the quantities required for germination.

Development of Genomics Tools

Genome Sequences and Phylogeny

Legume seed genomics is anchored in the pioneering studies on two model species, *M. truncatula* and *Lotus japonicus*. These two forage legumes were chosen primarily for their small genome sizes (*M. truncatula* 375 Mb (Young *et al.*, 2011), *L. japonicus* 470 Mb (Sato *et al.*, 2008)) and for their ease of genetic transformation (Barker *et al.*, 1990; Handberg and Stougaard, 1992). In the first instance, they were chosen to study the rhizobial symbioses they undergo and subsequently generally adopted as legume models.

A consortium was launched in 2003 to sequence the *M. truncatula* genome (http://www.medicagohapmap.org/?genome), and the Lotus genome sequencing project was started in parallel (http://www.kazusa.or.jp/lotus/index.html). Sato *et al.* (2008) reported 315.1Mb of sequences corresponding to 67% of the genome and covering ~90% of the gene space, and an update on this sequencing effort was presented subsequently by Sato *et al.* (2010). The Medicago genome sequence (Young *et al.*, 2011) reveals the presence of ~60,000 genes for both species at a density of 12.6/100 kb for *M. truncatula* compared with 17.4/100 kb for *L. japonicus*, the difference being due to the higher proportion of mainly retroposon-type repeated sequences in the *M. truncatula* gene-rich regions. The *M. truncatula* genome also contains a higher proportion of local gene duplication than the other sequenced legumes. The Medicago and Lotus genomic sequencing efforts both have followed the classical approach of assembling a BAC contig and sequencing BAC-by-BAC, with, for Medicago, alignment via optical mapping. More recently, massively parallel sequencing has been adopted to finish these sequences and to sequence other legumes including crop species. The first published genomic legume sequence was that of soybean (Schmutz *et al.*, 2010), and the *M. truncatula* sequence followed (Young *et al.*, 2011), with numerous other species genomes in

the pipeline (Table 10.1), the first to appear being that of the pigeonpea (Singh *et al.*, 2012). These sequences will fuel the generation of a new series of genomic resources, replacing those currently used based on unassembled genomic sequences and EST sequencing.

Sequence comparisons between *M. truncatula* and *L. japonicus* show the presence of extensive synteny, frequently extending over an entire chromosome arm. In contrast, a few regions show very little syntenic relationship, owing to extensive fine-scale rearrangements. The comparison also revealed the existence of an ancient whole-genome duplication (WGD) ~58 Myr. ago, which predated the Medicago-Lotus divergence (or predated speciation within the legume clade) and which is reflected in a corresponding second, lower level of synteny arising from matches between the paralogous segments between the two species (Cannon *et al.*, 2006). Both *M. truncatula* and *L. japonicus* belong to the Papilionid clade of the Fabaceae; the Medicago genus is in the Galegoid branch, including pea, clover, chickpea, pigeonpea, lentil, and broad bean, whereas Lotus is more closely related to the tropical legumes soybean and Phaseolus. The extensive synteny observed within these two groups (Hougaard *et al.*, 2008; Young and Udvardi, 2009; Young *et al.*, 2011) enables the transfer of knowledge from model species to crops. This process has been facilitated by the development of sets of gene-based anchor markers (Aubert *et al.*, 2006; Hougaard *et al.*, 2008) that are used to define syntenic "mileposts" when comparing related genetic maps. The phylogenetic similarity between *M. truncatula* and pea is reflected in close sequence similarity at the DNA level and in a high degree of synteny, which facilitates transfer of markers from the model to the crop species. In soybean, physical maps for *Glycine max* and its undomesticated ancestor, *Glycine soja* were built that will serve as a framework for ordering sequence fragments, comparative genomics, cloning genes, and evolutionary analyses of legume genomes (Ha *et al.*, 2012).

Legume Seed sRNA Databases

Although many miRNA sequencing projects are in progress, there are currently only two reports in the literature specifically on miRNAs expressed in legume seeds, on soybean (Song *et al.*, 2011) and *Phaseolus vulgaris* (Pelaez *et al.*, 2012). Because this situation is likely to change rapidly, we also provide references to plant miRNA prediction algorithms and databases that will be updated and that include seed-expressed miRNAs (Mhuantong *et al.*, 2009; Zhang *et al.*, 2010; Meng *et al.*, 2011; Bielewicz *et al.*, 2012). Legume-specific miRNA families have been identified in *M. truncatula* and *Phaseolus vulgaris* (Arenas-Huertero *et al.*, 2009; Jagadeeswaran *et al.*, 2009) but are not yet functionally characterized.

Reverse Genetics Resources Available for Model and Nonmodel Legumes

Numerous reverse genetics resources have been developed for legumes (Tadege *et al.*, 2009; Young and Udvardi, 2009) and the availability of genome sequences will accelerate the development of these tools in the near future. Two main complementary approaches were used . One approach produced point mutations throughout the genome that can be screened by Targeting Induced Local Lesions in Genomes (TILLING) in various protein domains of candidate genes. TILLING offers the opportunity to study *in vivo* protein function, identify enzyme active sites, and design novel alleles for quality improvement. The second approach created insertion or deletion mutations, which proved useful to understand the function of candidate genes when nonlethal phenotypes are obtained. TILLING resources have been created for many model and crop legumes, including pea (Dalmais

Table 10.1 Overview of Genomics Resources Available for Legume Species as of June 2012

Name	Synonym	Ploidy	Chromosome Number	Genome Size (Mbp)	ESTs (result of NCBI search May 2012)	Markers Resources	References for Marker Resources	Transcriptome Resources	Proteome Resources	Genome Resources
Acacia sp.		diploid	n = 13		11,569	Acacia mangium Willd: RFPL (219) SRR (33) Acacia senegal (L.) Willd: RAPD (10) ISSRs (5) SSRs (11)	Assoumane *et al.*, 2009 Butcher *et al.*, 2000 Josiah *et al.*, 2008			http://www.ukm.my/acacia/index.html
Arachis hypogaea (AB genome)	peanut, groundnut, earth nut, goober pea, mani, monkey nut, runner peanut, Spanish peanut, Valencia peanut, Virginia peanut	tetraploid	n = 10	2,813	178,490	SSRs (7482)	Pandey *et al.*, 2012	Zhang *et al.* (2012)	Guo *et al.* (2008), Kottapalli *et al.* (2008), Schmidt *et al.* (2009)	
Arachis duranensis (A-genome)						SSRs (1329) AFLP (148) SNPs (1144) RFLPs (121) AFLPs (148)	Pandey *et al.*, 2012			
Arachis ipaënsis (B-genome)					SSRs (598)	Pandey *et al.*, 2012				
Cajanus cajan	pigeon pea, kadios	diploid	n = 11	858	25,314	SSRs (46)	Odeny *et al.*, 2007 Saxena *et al.*, 2010	Dubey *et al.* (2011)		Singh *et al.* (2012)
Cicer arietinum	chickpea, Bengal gram, calvance pea, chestnut bean, chich, chich-pea, dwarf pea, garavance, garbanza, garbanzo bean, garbanzos, gram, gram pea, homes, hamaz, nohub, lablabi, shimbra, yellow gram	diploid	n = 8	740	44,157	RIL: C. arietinum (ICC 4958) × C. reticulatum (PI 489777): SSRs (311) SNPs (220) EST-SSRs (51) CISRs (87) CAPS (143)	Nayak *et al.*, 2010 Gujaria *et al.*, 2011	Garg *et al.* (2011)	Pandey *et al.* (2006, 2008), Bhushan *et al.* (2006)	
Cyamopsis tetragonoloba	guar bean, cluster bean, gawaar, gwaar ki phalli									
Dolichos lablab	hyacinth bean, bonavist, bataw, lablab									

Glycine max	soybean, soya, soyabean, edamame	diploid	n = 20	1,115	1,461,624	Glycine max var. Williams 82: SNPs (4991) SSRs (874)Glycine max (L.) Merr. : SNPs (2928) SSRs (1014) RFLP (709)	Schmutz *et al.*, 2010 Choi *et al.*, 2007	Vodkin *et al.* (2004), Choi *et al.* (2007), Libault *et al.* (2010), Mochida *et al.* (2010), Severin *et al.* (2010), Jones *et al.* (2010) Asakura *et al.* (2012), Sha *et al.* (2012)	Hajduch *et al.* (2005), Agrawal *et al.* (2008), Hajduch *et al.* (2011)	http://www.phytozome.net/soybean http://rsoy.psc.riken.jp/ http://soybase.org/ http://harvest.ucr.edu/
Lens culinaris	lentil, black lentil, brown lentil, green lentil, green mungbean, large-seeded lentil, red mungbean, small-seeded lentil, wild lentil, yellow lentil, adas, mercimek, messer, masser, heramame	diploid	n = 7	4,063	9,513	SSRs (46)	Hamwiech *et al.*, 2009	Kaur *et al.* (2011)	Scippa *et al.* (2010)	
Lotus japonicus		diploid	n = 6	472	242,432	SRRs (788) dCAPS (80)	Sato *et al.*, 2008		Dam *et al.* (2009), Nautrup-Pedersen *et al.* (2010)	http://www.kazusa.or.jp/lotus/ http://www.shigen.nig.ac.jp/bean/lotusjaponicus/
Lupinus angustifolius	Blue lupin	diploid	n = 20	740		SNP (8207)		Foley *et al.* (2011)		
Lupinus albus	sweet lupin	diploid	n = 25	455					Magni *et al.* (2007)	
Medicago sativa	alfalfa	tetraploid	n = 8		12,371					
Medicago truncatula		diploid	n = 8	~ 500	269,238	SNPs (3000000) SSRs (317)	Branca *et al.*, 2011 Mun *et al.*, 2006 Young *et al.*, 2009	Benedito *et al.* (2008), Kakar *et al.* (2008), Verdier *et al.* (2008) He *et al.* (2009)	Gallardo *et al.* (2007), Colditz and Braun (2010)	http://www.medicago.org/ http://www.medicagohapmap.org/index.php
Phaseolus acutifolius	Tepary bean, tepari bean									
Phaseolus lunatus	Lima bean, butter bean, patani									
Phaseolus vulgaris	commun bean, common field bean, kidney bean, navy, habichuela, snap bean	diploid	n = 11		123,988	EST-SSRs (302)	Garcia *et al.*, 2011	Yin *et al.* (2011)	De la Fuente *et al.* (2011)	

(*continued*)

Table 10.1 (*Continued*)

Name	Synonym	Ploidy	Chromosome Number	Genome Size (Mbp)	ESTs (result of NCBI search May 2012)	Markers Resources	References for Marker Resources	Transcriptome Resources	Proteome Resources	Genome Resources
Pisum sativum	pea, dry pea, Chinese pea, Chinese pea pod, Chinese snow pea, edible-podded pea, edible pod pea, podded pea, snap pea, snow pea, sugar snap pea, batani, chicharo, erbese, ater, pois, takamany borso, pisello, holoan, mange-tout, papdi	diploid	n = 7	4,778	18,576	SSRs (434) EST-SSRs (171)	Loridon *et al.*, 2005 Mishra *et al.*, 2012	Franssen *et al.* (2011), Kaur *et al.* (2012)	Bourgeois *et al.* (2011)	Bordat *et al.* (2011)
Trifolium pratense	red clover	diploid	n = 7	440	38,109	SSRs (1414) AFLP (181) RFP (204)	Isobe *et al.*, 2009			
Trifolium repens	white clover	allopoliploid	n = 8	956	46	SSRs (415) AFLP (283)	Khanlou *et al.*, 2011 Zhang *et al.*, 2007			
Vicia faba	broad bean, horse bean, fava bean, bell bean, field bean, tic bean	diploid	n = 6	~ 13000	5,415	EST-SSRs (11) ITAP (135) RADP (238) SSRs (191) AFLP (36)	Diaz-Ruiz *et al.*, 2010 Ellwood *et al.*, 2008 Gong *et al.* 2011	Kaur *et al.* (2012)		
Vicia sativa	vetch, commun vetch									
Vigna angularis	*Phaseolus angularis*, Adzuki bean, azuki bean, Adanka bean, danka bean	diploid	n = 11	540	25	SSRs (191 AFLP (36)	Kaga *et al.*, 2008			
Vigna unguiculata	*Vigna sinensis*, cowpea, asparagus bean, black eyed pea, black eyed bean, crowder pea, field pea, southern pea, frijole, lobhia, kibal, nieve, paayap	diploid	n = 11	620	187,487	SNPs (298) SSRs (102)	Gupta *et al.*, 2010 Timko *et al.*, 2008	Diouf *et al.* (2011)	Nogueira *et al.* (2007)	
Vigna radiata	*Phaseolus aureus*, mung bean, black dahl, black gram, black mung, golden gram, gram bean, green gram, mungo, red mung bean, urd, chop suey bean http://www.cropsreview.com/grain-legumes.html	diploid	n = 11	580	829	RIL: Berken × ACC41 SSRs (97) RFLPs (76) RAPDs (4) STSs (2)	Zhao *et al.*, 2010	Moe *et al.* (2011)		

Note. The sections on transcriptomics and proteomics resources are limited to analyses of seed tissues.

et al., 2008), soybean (Cooper *et al.*, 2008), *M. truncatula* (Le Signor *et al.*, 2009), *L. japonicus* (Perry *et al.*, 2003), Cicer (Tadege *et al.*, 2009; Tapan *et al.*, 2012), Phaseolus (Porch *et al.*, 2009), peanut (Knoll *et al.*, 2011), and pigeonpea (Varshney *et al.*, 2010).

De-TILLING (Rogers *et al.*, 2009) is an adaptation of the TILLING procedure that was used to screen deletions in *M. truncatula* created by fast neutron mutagenesis. A population of 156,000 *M. truncatula* plants was structured as 13 towers each representing 12,000 M2 plants. Three-dimensional pooling allows efficient location of mutants from within the towers. Mutants were detected from this population at a rate of 29% using five targets per gene.

Insertion mutant resources have been created for *M. truncatula* by incorporating the retrotransposon Tnt1 from tobacco (d'Erfurth *et al.*, 2003). These resources have been developed by the Noble foundation, which provides a reverse genetic platform permitting screening of 12,000 lines, a total of 300,000 insertions, either by an in silico screen of sequenced Tnt1 insertion borders or by PCR on DNA bulks from the collection (Tadege *et al.*, 2008; http://bioinfo4.noble.org/mutant/). Endogenous retrotransposons have been mobilized in *M. truncatula* (Rakocevic *et al.*, 2009) and in *L. japonicus* (Fukai *et al.*, 2012) and form the basis of further insertion mutant collections. In contrast, this approach, to our knowledge, has not been successfully applied to major crop legumes for which no insertion mutant collections exist.

Virus-induced gene silencing (VIGS) is another strategy that has been developed for functional genomics in model and crop legumes by at least three laboratories (Constantin *et al.*, 2004; Igarashi *et al.*, 2009; Varallyay *et al.*, 2010). The most promising approach uses agro-infection to furnish virus during inoculation. These methods involve transient transformation, generally of an RNAi construct, and are particularly suited to the rapid detection of qualitative changes in plant morphology. Their use for specifically studying legume seed phenotypes has not been reported so far. Nishizawa *et al.* (2010) took an interesting alternative route by transforming somatic embryos with a vector containing an antisense construction for beta-conglycinin and observed reduced conglycinin expression in transformed embryos.

Applications of Genomics Tools to Legume Seed Biology

Characterizing the Proteome and Transcriptome of Developing Seeds

Large-scale proteomics and transcriptomics analyses of seed gene expression have been carried out on several legume species, including both models and crops. Lei *et al.* (2011) created a useful legume-specific protein database (LegProt, http://bioinfo.noble.org/manuscript-support/legumedb/) that regroups much of the proteomics information obtained, whereas Li *et al.* (2012) set up Legume IP, a platform for transcriptomics data from the model legume species. In this chapter, we have selected studies from some of the best-researched legumes.

In soybean, a high-throughput proteomic approach using two-dimensional gel electrophoresis (2DGE) and matrix assisted laser desorption ionization (MALDI-TOF) was employed to determine the expression profile and identity of 679 proteins during seed filling (Hajduch *et al.*, 2005). There were 14 major functional categories. Proteins involved in metabolism, protein destination and storage, metabolite transport, and disease and defense were the most abundant. For each functional category, a composite expression profile was obtained. An overall decrease in metabolism-related proteins versus an increase in proteins associated with destination and storage was observed during seed filling. A user-intuitive database (http://oilseedproteomics.missouri.edu) was developed to access these data for soybean and other oilseeds (currently oilseed rape, *Arabidopsis,* castor

bean). More recently, this group combined 2DGE and semicontinuous multidimensional protein identification technology (Sec-MudPIT) coupled with liquid chromatography–mass spectrometry, which decreased the redundancy of the sequence data obtained (Agrawal *et al.*, 2008). Comparison of the dataset with a parallel study of rapeseed (*Brassica napus*) (Hajduch *et al.*, 2011) uncovered an increased expression of glycolytic and fatty acid biosynthetic proteins in rapeseed compared with soybean, which the authors suggest could be responsible for higher oil in rapeseed owing to an increased commitment of hexoses to glycolysis and eventually to *de novo* fatty acid synthesis pathways.

More recently, a 27K soybean microarray resource developed by Vodkin *et al.* (2004), which included immature cotyledon ESTs, was used for monitoring seed development by Jones *et al.* (2010). These authors first noted the high abundance of transcription factor transcripts in the mature seed, which are thought to be stored in the dry seed in preparation for germination. An Affymetrix genechip soybean genome array (http://www.affymetrix.com) bearing 37,500 transcript sequences, plus 15,800 transcripts for *Phytophthora sojae* and 7500 for *Heterodera glycines* (cyst nematode pathogen) transcripts was also made available and was used by Asakura *et al.* (2012) to profile soybean transcription throughout seed development. High-throughput sequencing was used to analyze normalized cDNA libraries from a series of stages of soybean seed development (Sha *et al.*, 2012). Soybean transcriptome data are accessible at SoyXpress (Cheng and Stromvik, 2008) and at LegumeIP, a site that integrates information from several model legumes (Li *et al.*, 2012). Detailed seed transcriptome information for soybean and *Phaseolus coccineus* is also available at http://seedgenenetwork.net/ site. This project has exploited laser capture microdissection (LCM) to obtain RNA samples from many component tissues of the seed that have been converted to cDNA and subjected to high-throughput sequencing (Le *et al.*, 2007). LCM allows a much finer assignment of gene expression profile to cell type than is possible with hand-dissection. Le *et al.* (2007) identified many region-specific transcripts and could show that coregulation was higher for genes expressed from the same region. They also undertook the first detailed study of regulation of suspensor gene expression.

Dam *et al.* (2009) characterized the development of seeds in the model legume *Lotus japonicus*. Protein, oil, starch, phytic acid, and ash contents were determined, which indicated that the composition of mature Lotus seed is more similar to soybean than to pea, consistent with its phylogenetic proximity to the Phaseoleae tribe rather than the Galegoid tribe. Two-dimensional polyacrylamide gel electrophoresis and gel-based liquid chromatography–mass spectrometry were used to identify proteins. Five legumins, LLP1 to LLP5, and two convicilins, LCP1 and LCP2, were identified by MALDI-TOF mass spectrometry. For two distinct developmental phases, seed filling and desiccation, 665 and 181 unique proteins corresponding to gene accession numbers were identified for the two phases. All of the proteome data are available at http://www.cbs.dtu.dk/cgi-bin/lotus/db.cgi (Lotus Experiment Database). A second Web interface (http://bioinfoserver.rsbs.anu.edu.au/utils/PathExpress4legumes/) was set up to collect and collate all protein identifications for Lotus, Medicago, and soybean seed proteomes.

The same group extended their analysis and included a comparative analysis of the pod proteome, identifying 604 pod proteins and 965 seed proteins, including 263 proteins distinguishing the pod (Nautrup-Pedersen *et al.*, 2010). The complete dataset is publicly available at the Lotus Experiment Database website, where spots in a reference map are linked to experimental data, such as matched peptides, quantification values, and gene accessions. Identified pod proteins represented enzymes from 85 different metabolic pathways, including storage globulins and a late embryogenesis abundant protein. In contrast to seed maturation, pod maturation was associated with decreasing total protein content, especially proteins involved in protein biosynthesis and photosynthesis. Proteins

detected only in pods included three enzymes participating in the urea cycle and four in nitrogen and amino group metabolism, highlighting the importance of nitrogen metabolism during pod development. Additionally, five legume seed proteins previously unassigned in the glutamate metabolism pathway were identified, consistent with the important role of the pod in nutrient, and particularly N, relocation, and in contrast to the N-deposition occurring in developing seeds. A Lotus gene expression atlas (LjGEA) has been set up, similar to that available for Medicago and including 79 tissues and five stages of seed development (J. Verdier, personal communication).

To investigate processes leading to desiccation tolerance in seeds, 16k-microarrays were used to monitor changes in the transcriptome of desiccation-sensitive radicles of *Medicago truncatula* seeds during incubation in a polyethylene glycol solution (Buitink *et al.*, 2006). During the incubation, >1300 genes were differentially expressed. A large number of genes involved in carbon metabolism are expressed during the re-establishment of desiccation tolerance. Quantification of carbon reserves confirms that lipids, starch, and oligosaccharides were mobilized, coinciding with the production of sucrose during the early osmotic adjustment. Several regulatory genes typically expressed during abiotic/drought stresses were also upregulated during maturation, arguing for the partial overlap of abscisic acid–dependent and abscisic acid–independent regulatory pathways involved in both drought and desiccation tolerance.

The availability of *M. truncatula* genomic sequence has permitted the development of an Affymetrix chip representative of the transcriptome (Benedito *et al.*, 2008). The data obtained with this array, including samples taken at six time points through seed development, have been assembled and are publicly available on the Medicago Gene Atlas site (He *et al.*, 2009). The relative insensitivity of chip hybridizations has motivated the development of a qRT-PCR platform for *M. truncatula* transcription factor (TF) genes (Kakar *et al.*, 2008). This platform was used by Verdier *et al.* (2008) to analyze the expression of >700 *M. truncatula* genes encoding putative TFs throughout seven stages of seed development. By applying a k-means clustering to the expression data, six classes of TF profiles could be identified, corresponding to the developmental stages. Four different TF classes correlated with the start of storage protein mRNA synthesis, vicilin-type protein synthesis, legumin A synthesis, and legumin K synthesis, these events being sequential in legume seed development. In complement, by purifying nuclei from *M. truncatula* developing seeds at the beginning of the seed filling stage, Repetto *et al.* (2008) obtained sequence identifications of >100 nuclear-localized proteins. Most were ribosomal proteins or other proteins involved in rRNA synthesis in the nucleolus, but numerous other chromatin-related proteins and transcription factors were also represented.

First attempts have been made to compare proteomics with transcriptomics data from developing *M. truncatula* seeds by Gallardo *et al.* (2007), who classified genes according to kinetic profiles of expression. Although for many genes the protein encoded had a temporal expression profile similar to that of the corresponding transcript, there were some exceptions. In cases where transcript accumulation is not accompanied by the corresponding protein, several explanations are possible. The most likely is that there is a translational control on protein accumulation operating. This may be analogous to that observed for stored mRNAs accumulated in maturing seeds for use during germination. miRNAs found in developing seeds (see later) may be implicated in translational control of some mRNAs. The protein may also be rapidly degraded or processed and not be detected in its intact form. Where the protein appears to accumulate at stages where there is little of the corresponding mRNA, it is assumed that the protein is stable, but the mRNA is unstable and rapidly turned over. A double-labeling allowing measuring of the *de novo* synthesis of mRNA and of proteins would provide additional information useful to understand the regulation of seed protein accumulation.

Seed Metabolomics

Currently, publications on legume seed metabolomics are essentially limited to applications of metabolomics to understand the effect of single-gene mutations on seed composition. An example of this type of publication is Vigeolas *et al.* (2008), who examined the effect of reduced PA2 albumin accumulation on pea seed composition. In an analysis by chromatography–mass spectrometry of soluble metabolites in the line lacking PA2 albumin, there was a shift in the relative proportions of carbon metabolites from sugars to amino acids, whereas later in seed development, sugar levels were similar to the wild-type line, but there were increases in several amino acids, particularly arginine, a precursor of spermidine, which was accompanied by decrease in the latter. In addition, there was an overall increase in protein content in mature seeds. Consistent with this result, arginine decarboxylase (ADC) activity was reduced in PA2 minus lines. However, the ADC locus was unlinked to that of PA2, ruling out a direct effect of the PA2 locus mutation on ADC.

Frank *et al.* (2009) used a chromatography–based approach to compare the metabolite profiles of two low phytic acid (lpa) soybean mutants and their wild-types. Both the lpa mutants had major phytic acid reductions. For Gm-lpa-TW75-1, decreased accumulation of lower inositol phosphates was also observed, consistent with a mutation in *myo*-inositol phosphate synthase. Consideration of the metabolic changes observed for Gm-lpa-ZC-2 (accumulation of lower inositol phosphates and increased *myo*-inositol contents) indicated a mutation event affecting the latter biosynthetic steps leading to phytic acid, possibly in the inositol phosphate kinase gene. The study demonstrated the applicability of metabolite profiling for the detection of changes in the metabolite phenotype induced by mutation breeding.

Modifying Seed Characteristics by Manipulating Gene Expression

Protein Composition

Several groups have obtained soybean mutants defective in storage protein accumulation in the developing seed (Takahashi *et al.*, 1994; Hayashi *et al.*, 1998; Krishnan *et al.*, 2001; Kim *et al.*, 2008; Hayashi *et al.*, 2009; Lee *et al.*, 2011). These mutants include trans-acting dominant and recessive loci controlling 7S Globulin (beta-conglycinin) accumulation, and cis-acting alleles at these loci (Teraishi *et al.*, 2001; Jegadeesan *et al.*, 2012).

To analyze the rate-limiting role of amino acids for seed protein synthesis, a *Vicia faba* amino acid permease, VfAAP1, has been ectopically expressed in pea (*Pisum sativum*) and *Vicia narbonensis* seeds under the control of the legumin B4 promoter (Rolletschek *et al.*, 2005). In mature seeds, starch is unchanged, but total nitrogen is 10%–25% higher, which affects mainly globulin, vicilin, and legumin, rather than albumin synthesis. Pea seeds overexpressing a sucrose transporter also show stimulated storage protein biosynthesis at the level of transcripts, proteins, and protein bodies. It was concluded that sucrose functions as both a signal and a fuel to stimulate storage protein accumulation (Rosche *et al.,* 2005). By RNA interference, Schmidt *et al.* (2011) suppressed the synthesis of soybean (Glycine max) glycinin and conglycinin storage proteins. The storage protein knockdown (SP−) seeds resemble the wild-type and maintain wild-type levels of protein and storage triglycerides. The SP− soybeans were evaluated with systems biology techniques of proteomics, metabolomics, and transcriptomics using both microarray and RNA-Seq. Proteomic analysis shows that rebalancing of protein content largely results from the selective increase in the accumulation of only a few proteins: Kunitz trypsin inhibitor, soybean lectin, and the immunodominant soybean allergen P34 or Gly m Bd 30k. A similar set of proteins greatly increases in accumulation in a cross

between two soybean lines that carry conglycinin and glycinin null alleles (Takahashi *et al.*, 2003). The rebalancing of protein composition occurs with small alterations to the seed's transcriptome and metabolome.

Major legume seed proteins are very low in cysteine and tryptophan, the latter being an essential amino acid. Efforts have been made to manipulate amino acid composition, to increase the accumulation of sulfur-rich proteins or to introduce a sulfur-rich foreign protein (Krishnan, 2005; Kita *et al.*, 2010). Insertion by transgenesis of a protein "sink" rich in cysteine residues results in a decrease in other sulfur compounds, such as free sulfur amino acids and glutathione (Tabe and Droux, 2002). Generally, only modest increases in seed cysteine content were obtained, suggesting amino acid supply may be limiting. To address this possibility, Tan *et al.* (2010) increased phloem transport of *S*-methylmethionine, by expressing a yeast *S*-methylmethionine transporter from the AAP1 promoter in transgenic pea. The transgenics had increased plant vigor and increased phloem transport of both sulfur and nitrogen. Seed number was also increased, but the seed sulfur content was unchanged. Tabe *et al.* (2010) demonstrated that cysteine biosynthesis in legumes can be modified by manipulating sulfur assimilatory pathways. More recently, Kim *et al.* (2012) overexpressed an enzyme in the cysteine biosynthetic pathway, O-acetylserine sulfhydrylase, in transgenic soybean. The transformed seeds had significantly increased free and protein-bound cysteine contents. Elevated levels of methionine in seeds of narbon bean (*Vicia narbonensis* L.) was also obtained by generating double transformants, which express a methionine-rich storage protein and a feedback-insensitive bacterial aspartate kinase. Aspartate kinase is an enzyme in the methionine biosythesis pathway, which in plants is feedback-inhibited by several intermediate precursors (Demidov *et al.*, 2003).

Studies on the regulation of storage protein gene expression in the common bean (*Phaseolus vulgaris*) date from the early 1980s, making it one of the best-characterized systems in terms of promoter-TF interactions despite the absence for most of this period of a homologous stable genetic transformation protocol. Of particular interest is the existence of bean lines defective in accumulation of major storage protein classes (Blair *et al.*, 2007), which have been used as the basis for improving seed amino acid composition (Taylor *et al.*, 2008). Marsolais *et al.* (2010) and Yin *et al.* (2011) have carried out a study of common bean seed composition for mutants defective in storage protein accumulation. Yin *et al.* (2011) generated an EST collection from RNA of developing common bean seeds and used this database to investigate the basis for elevated sulfur content in lines lacking major sulfur-poor proteins. They observed increases in sulfur-rich proteins and in starch and raffinose metabolic enzymes and a downregulation of proteins of the secretory pathway. The reduced levels of seed storage proteins were accompanied by an increase in sulfur amino acid content in genetically related lines. Reduced phaseolin, phytohemagglutinin, and arcelin were mainly compensated by increases in legumin, the mannose lectin FRIL, and α-amylase inhibitors. The increased S-amino acid content was due principally to legumin, albumin-1 and albumin-2 and defensin, whereas gamma-glutamyl-S-methyl-cysteine was reduced. Traffic through the protein secretory pathway was disturbed, as shown by changes in various proteins implicated in its functioning.

Oil Composition

In addition to modification of protein content and composition, there is interest in increasing soybean oil content and modifying its composition (see Chapter 11). Wang *et al.* (2007) screened soybean Dof type transcription factors that were expressed during seed development and found two of these that increased seed fatty acid accumulation when overexpressed in *Arabidopsis*. Several groups have identified mutations in the omega-6 fatty acid desaturase gene, which converts linoleic acid to

oleic acid. This is of interest for providing high oleic acid oils. Dierking and Bilyeu (2009) screened mutations in the omega-6 fatty acid desaturase gene, FAD 2-1A, from a Soybean TILLING collection and obtained a mutant allele affected in a conserved region of the enzyme. Seeds of this mutant had an altered fatty acid composition, with increased proportion of oleic acid and decreased linoleic acid, as predicted.

Manipulation of Starch Deposition

The effect of repressing ADP-glucose pyrophosphorylase (AGP) synthesis by RNAi in pea seeds was analyzed using transcript and metabolite profiling to monitor the effects that reduced carbon flow into starch has on carbon-nitrogen metabolism and related pathways (Weigelt *et al.*, 2009). Repressing AGP has several consequences. It appears to enhance provision of precursors such as acetyl-coenzyme A and organic acids, which promote amino acid and storage protein biosynthesis as well as pathways fed by cytosolic acetyl-coenzyme A, such as cysteine biosynthesis and fatty acid elongation/metabolism. The resulting higher nitrogen (N) demand depletes transient N storage pools, specifically asparagine and arginine, and leads to N limitation. Seed size is reduced, although protein content is increased. Increased sugar accumulation appears to stimulate cell proliferation, and deregulation of starch biosynthesis resulted in increased mitochondrial metabolism and osmotic stress. Although multiple stress responses are elicited, these occur in a controlled fashion, and premature senescence or apoptosis is not seen.

Vriet *et al.* (2010) have obtained mutations in genes involved in starch biosynthesis from a *Lotus japonicus* TILLING collection. Although this article is primarily focused on starch metabolism in vegetative tissues, it does report on the seed phenotype of a mutant in *GWD* (glucan water dikinase), an enzyme involved in starch degradation. The homozygous mutant plant showed a strong maternal effect with a high proportion of aborted seed and pods, showing the importance of glucan water dikinases in starch degradation and the importance of this process in seed development, presumably in remobilizing carbon in maternal tissues, there being no starch accumulation in the mature seed of *L. japonicus*.

Antinutritionals

Among the main breeding objectives for pulse legumes is the elimination by genetic selection or genetic manipulation of certain seed components that reduce digestibility. In most cases, progress has been made by forward genetic screens. Null alleles of the major trypsin inhibitor *Tri* genes of pea have been routinely selected for Pea breeding in recent years, based on a PCR screen (Page *et al.*, 2002; Chinoy *et al.*, 2011). A naturally occurring mutant allele in pea of the poorly digested and possibly allergenic PA2 albumin is also available (Vigeolas *et al.*, 2008). However, the removal of these proteins has to be balanced against their proposed contributions to seed insect and fungal resistance (Srinivasan *et al.*, 2005; Da Silva *et al.*, 2010). Members of the raffinose family oligosaccharides (RFOs) accumulate in maturing seeds of many legumes, where they appear to contribute to desiccation tolerance. RFOs are poorly digested by the intestinal flora of monogastric animals and cause flatulence – hence efforts to eliminate their production. Dierking and Bilyeu (2009) screened a soybean TILLING population for EMS mutations in the Raffinose Synthase gene *rs2*. They obtained a mutant that had 24% stachyose and 37% raffinose levels compared with wild-type, accompanied by an increase in seed sucrose concentration. The residual RFOs are probably due to the presence of a second RS gene. Polowick *et al.* (2009) took a different, transgenic, approach by overexpressing an alpha-galactosidase from Coffea in pea seeds. Using this strategy, they obtained similar reductions in RFOs to those observed in the soybean *rs2* TILLING mutant.

Understanding Regulation of Gene Expression in Seeds

Functional analyses of legume seed promoters began in the 1980s with work on the phaseolin (common bean storage protein) gene from Hall's laboratory (Slightom *et al.*, 1983). The phaseolin promoter was functional in the heterologous tobacco system, where it conferred embryo-specific expression (Senguptagopalan *et al.*, 1985). There followed detailed promoter dissection that identified several cis-acting elements in the phaseolin promoter and in that of an unidentified seed protein gene from *Vicia faba* USP (Fiedler *et al.*, 1993). Hall's group showed that activation of the phaseolin promoter required at least two steps: the binding of an activator transcription factor (TF), PvALF, that modified chromatin conformation on the promoter and an ABA-dependent step of gene activation (Li *et al.*, 1999). Subsequently, these two steps were shown to correspond to histone lysine acetylation and methylation events in the associated nucleosomes on the Phas promoter that potentiate gene activation (Ng *et al.*, 2006). More comprehensive analyses of the TFs interacting with seed-expressed promoters in legumes have been made possible by the availability of transcriptome data.

In contrast to studies on maize or *Arabidopsis*, most legume TFs involved in seed development and seed filling have not been identified via screening for defective seed mutant phenotypes but initially by sequence homologies with previously characterized TFs. To restrict the number of TFs being analyzed, generally only legume TFs coexpressed (in time and in tissue) with putative target promoters have been studied. More recent global transcriptomics analyses have confirmed the overall similarity of the seed TF complement of *M. truncatula* with that of *Arabidopsis* seeds, consistent with their similar ontogenesis and morphologies, although very few functional analyses have yet been reported (Udvardi *et al.*, 2007). Verdier *et al.* (2008) analyzed the expression by q-RT-PCR of >700 *M. truncatula* genes encoding putative TFs throughout seven stages of seed development. Of 169 TFs expressed during seed filling, the tissue specificity within the seed was examined for the 41 most highly expressed sequences. To identify possible target genes for these TFs, the data were combined with a microarray-derived entire transcriptome dataset. This study identified 17 TFs preferentially expressed in individual seed tissues and 135 corresponding coexpressed genes, including possible targets. Some of the TFs, which coexpressed with storage protein mRNAs, were homologous to those already known to regulate seed storage protein synthesis in *Arabidopsis*. Putative orthologues of the "master regulators" of seed development LEC1, LEC1-LIKE, FUS3, and ABI3 could be identified in *M. truncatula*, showing an overall conservation of gene-specific transcription factor sequences in the seeds of the two genera. However, an obvious candidate for a LEC2 orthologue was not found in *M. truncatula*. *M. truncatula*, similar to other legumes, has a delayed expression of the legumin-class storage proteins with respect to the vicilin class of storage proteins, and a class of TFs could be specifically assigned to this phase of expression.

An alternative approach to narrowing down the gene candidates for encoding a given trait is to look for genetic linkage. Gillman *et al.* (2011) used genetic mapping and candidate gene association in a RIL population and a panel of soybean lines with defined coloration (seed coat and hilum, pubescence, and flower) to determine the R gene controlling black or brown seed coat coloration secondary to anthocyanin derivatives in soybean to be an R2R3 MYB gene (Glyma09g36990). Similarly, Verdier *et al.* (2012) have identified an R2R3Myb, MtrPAR1, which controls proanthocyanidin production in *Medicago truncatula* seed coats. By overexpressing this TF in transgenic hairy roots, they were able to accumulate proanthocyanidin ectopically in the roots. These studies may offer potential for eliciting proanthocyanidin accumulation in vegetative tissues of forage crops, such as Lucerne/alfalfa, to combat pasture bloat in ruminants.

In contrast to *Arabidopsis*, many crop legumes (pea, faba bean, common bean, lupin) accumulate starch in the mature seed. It will be of interest to see whether dedicated TFs control this process.

Identifying Loci Controlling Seed Traits

The *M. truncatula* model has been exploited to identify genes important for seed maturation (Vandecasteele *et al.*, 2011). The maturation of the seed comprises several distinguishable stages: acquisition of desiccation tolerance, acquisition of seed dormancy, determination of longevity, and determination of germinative seed vigor. In contrast to *Arabidopsis*, maturing legume seeds accumulate large quantities of raffinose family oligosaccharides (RFOs), which are thought to play a role in these processes and provide a useful complement to studies on other plant models. Vandecasteele *et al.* (2011) used two recombinant inbred line (RIL) populations of *M. truncatula* to map QTL for physiological traits related to germination. Several of the QTLs were found colocated with QTL controlling the Sucrose/RFO ratio and suggested that a decreased sucrose/RFO ratio might be a positive determinant of seed vigor. One of these populations, named LR4, has been used to reveal a large number of QTL controlling seed weight and composition (K. Gallardo, personal communication) and to map QTL for seed mineral concentration (Sankaran *et al.*, 2009). The concentrations and content of seed Ca, Cu, Fe, K, Mg, Mn, P, and Zn was determined in the LR4 population, leading to the identification of 46 QTLs for seed mineral concentration and of 26 QTLs for seed mineral content. The availability of genomic sequences in this species will facilitate the positional cloning of genes controlling these traits. The Medicago HapMap project (http://www.medicagohapmap.org/) is sequencing 384 inbred lines using Solexa technology to detect SNPs at high density and provide a platform for association mapping.

Several laboratories have used molecular markers to carry out QTL analyses of soybean seed size and composition, including Li *et al.* (2007), who identified loci controlling protein and oil content, and Teng *et al.* (2009), who identified loci controlling seed weight. The availability of EST sequences and high-throughput sequencing of the *G. max* genome have permitted the development of high-density linkage maps and a panel for QTL mapping using SNPs (Choi *et al.*, 2007; Hyten *et al.*, 2010). A PQL (protein quantity loci) analysis of mature pea seeds was performed that identified QTLs for several hundreds of protein spots detected on 2D gels from 157 recombinant inbred lines (Bourgeois *et al.*, 2011). Most protein quantity loci mapped in clusters, suggesting that the accumulation of the major storage protein families was under the control of a limited number of loci. Some loci controlled both seed protein composition and protein content, and a locus on LGIIa appears to be a major regulator of protein composition and of *in vitro* protein digestibility. With the objective of identifying genes controlling such traits in grain legumes, the power of the translational genomics approach from highly characterized model plants to poorly characterized crop plants was underlined by Bordat *et al.* (2011). This approach, which integrates genetics and genomics data, provides a valuable source of markers to saturate a zone of interest. A broader view of pea genome evolution was obtained by revealing syntenic relationships between pea and sequenced genomes. Blocks of synteny were identified that gave new insights into the evolution of chromosome structure in Papillionoids and Eudicots.

Future Challenges

Network Construction

The seed represents the sum of the metabolic activities of all the plant organs contributing to its production. This also applies to the embryo and endosperm, which depend on the other seed tissues for their metabolite supply and development. Although it is simple to identify loss-of-function

mutations affecting seed development, the relationship between the genes encoded is not always evident, partly because of the interactions between the component tissues.

To establish connections between interdependent gene products, many groups have developed methods for identifying networks of interaction. This approach is likely to be crucial for seed biology in general and important for legume seed biology where the loci affecting nutrient balance in the whole plant are particularly important. The method of Bassel *et al.* (2011) exploited 138 previously obtained transcriptomics datasets on gene expression during *Arabidopsis* germination. A coexpression analysis using significance analysis of microarrays (SAM) (Tusher *et al.*, 2001) revealed two distinct networks of coexpression, for genes expressed in germinating and nongerminating seeds. By analyzing the highest degree nodes (hubs) of the network, these authors were able to identify a series of putative regulators of seed germination. The same methodology is being used for *Medicago truncatula* seed expression datasets (J. Buitink, personal communication). Once nonconditional sources of information are used, it becomes necessary to distinguish the mode of interaction of network elements. One possible approach was taken by Junker *et al.* (2010), who adopted engineering terminology that allows the representation of a series of operationally different relationships.

Integrating Physiological Modeling

To incorporate physiological and metabolic data into functional seed models, it is necessary first to acquire data in three dimensions within the developing seed and second to conceive programs capable of handling and representing the data (Gubatz *et al.*, 2007). Data in three dimensions can be approximated by using optical sections, for example, based on serial optical (confocal microscopy) or derived from mechanical sections. The datasets can be obtained for macromolecular seed constituents such as specific proteins by immunocytochemistry or for transcripts by in situ hybridization, and for insoluble carbohydrates or lipids as well as for cellular structures using staining methods. Advances in nuclear magnetic resonance imaging have extended this noninvasive 3D technique to allow for the first time the identification of metabolite distributions within the legume seed (Melkus *et al.*, 2009) (see Chapter 13). The adoption of this type of method, along with high-throughput enzyme assays, is vital for confirming the occurrence and extent of metabolic processes that are often only inferred from gene expression data.

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11 Cotton Fiber Genomics

Xueying Guan and Z. Jeffrey Chen

Introduction

Cotton is the largest source of renewable textile fiber and an important oil crop. Cotton fibers make comfortable apparel, beautiful home furnishings, durable paper money such as U.S. dollars (75% cotton and 25% linen), and innovative products such as plastics and digital screens. World consumption of cotton fiber is ~115 million bales or ~27 million metric tons per year (National Cotton Council, Cotton Council International, http://www.cottonusa.org/index.cfm). Cotton is also a major oilseed crop. Because of its flavor stability, cottonseed oil is used for making mayonnaise, salad oil, and salad dressing. Nonhydrogenated cottonseed oil is also used as a deep frying medium to reduce trans fatty acid content in French fries (Daniel *et al.*, 2005). However, raw cottonseed oil has to be extensively treated to reduce the level of gossypol, the consumption of which may produce toxic effects in humans, monogastric animals, and insects (Sunilkumar *et al.*, 2006; Mao *et al.*, 2007). In addition, cottonseed meal (after removal of oil) is the second most abundant plant protein feed available throughout the United States, after soybean meal (National Cottonseed Products Association, www.cottonseed.com). It is usually used for animal feed and in organic fertilizers. Modifying cottonseed for food and feed could profoundly enhance nutrition and livelihoods of millions of people in food-challenged economies.

Cotton fiber is seed hair or trichome and an ideal model for the study of plant cell elongation and cell wall and cellulose biosynthesis (Delmer, 1999; Kim and Triplett, 2001). Each seed produces ~25,000 cotton fibers, which represent 25%–30% of epidermal cells on a cotton ovule (Basra and Malik, 1984; Wilkins and Jernstedt, 1999). Each cotton fiber is a singular and elongated cell from the epidermal layer of the ovule and can reach 1–3 cm in length, which is probably the longest plant cell type (Kim and Triplett, 2001). The fiber is composed of nearly pure cellulose, the largest component of plant biomass. The completion of cotton fiber development and maturation takes 50–60 days, from fiber cell initiation, cell elongation, primary cell wall and secondary cell wall formation, to dehydration and maturation.

Cotton is a good model for understanding the functional and agronomic significance of polyploidy and genome size variation within the *Gossypium* genus. Fiber trait is a good example of natural and artificial selection during cotton genome evolution. Greater than 95% of the annual cotton crop worldwide is upland or American cotton (*Gossypium hirsutum* L.), and the extra-long staple or Pima cotton (*Gossypium barbadense* L.) accounts for <2% (National Cotton Council, 2010). Genetic and genomic improvement of cotton fiber production and processing will ensure that this

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natural-renewable product will be competitive with petroleum based synthetic fibers. This chapter reviews molecular and genomic studies on cotton fiber development with an emphasis on cotton fiber initiation.

Cotton Fiber Development

Cotton fibers are unicellular seed trichomes differentiated from the ovule epidermal layer. Fiber development is a highly regulated process that includes four overlapping phases termed fiber initiation, fiber elongation, secondary wall biosynthesis, and maturation (Figure 11.1A) (Basra and Malik, 1984; Tiwari and Wilkins, 1995; Wilkins and Jernstedt, 1999; Kim and Triplett, 2001). In *G. hirsutum*, lint fibers develop before or on the day of anthesis (defined as 0 days post anthesis [DPA]) (green-colored cells, Figure 11.1D), and the process is quasisynchronized in each developing ovule and among ovules within each ovary (boll) (Figure 11.1B). Fuzz fiber development usually occurs in a later stage but varies among genotypes (Figure 11.1E); fuzz fibers cease developing shortly after (brown-colored cells, Figure 11.1E) and remain attached to seeds after ginning (Figure 11.1F, lower middle). During ginning, lint fibers are spun into yarn, and fuzz fibers become trash. In fiberless mutants such as naked seed (*N1N1*), fiber initiation is delayed (Figure 11.1B), and no fuzz fiber is formed (Figure 11.1F, lower right corner) (Lee *et al.*, 2006). The fiber initials for lint fiber continue to grow without cell division for ~16–25 days (fiber elongation), concurrently with the biosynthesis of cellulose, which is the primary composition of cell walls in fibers (Figure 11.1C) (John and Crow, 1992; Kim and Triplett, 2001). The elongated fiber cells can range in length from 1–3 cm (Figure 11.1F) (Kim and Triplett, 2001). Fibers in *G. barbadense* may reach lengths of 6 cm. Fiber cells from *G. hirsutum* range in diameter from 11–22 μm, and the fiber length is 1000–3000 times longer than their width. Finally, fiber cells start to mature at ~50–60 DPA when cotton bolls open (Figure 11.1C). Long mature fibers (lint fibers) can be detached from the seeds and used for textile and other applications.

Among four distinctive stages of fiber development, fiber initiation and fiber elongation are extensively investigated. Despite recent advances, the molecular basis of fiber initiation remains poorly understood. Fiber initiation is a quasisynchronous process that occurs rapidly right after anthesis. Fiber cell differentiation from protodermal cells may start even before anthesis (about 2–3 days before anthesis) (Graves and Stewart, 1988a, 1988b). After application with auxin and gibberellic acid (GA), ovules harvested at 3 DPA and 2 DPA can produce fiber on culture media (Graves and Stewart, 1988b). Cell fate determination (fiber cells or nonfiber cells) precedes the formation of morphologically visible fiber cell initials (Figure 11.1D and E), and ~15%–25% of the epidermal cells become spinnable fibers (Kim and Triplett, 2001; Lee *et al.*, 2006). Fiber initiation and elongation are orchestrated in each fiber cell through changes in gene expression and intercellular signaling pathways (Lee *et al.*, 2006). Fiber elongation requires an increased level of protein synthesis for rapid cell growth and development (Basra and Malik, 1984).

Roles for Transcription Factors in Development of *Arabidopsis* Leaf Trichomes, Seed Hairs, and Cotton Fibers

Together with *Arabidopsis* leaf trichome, root hair, and pollen tube (Larkin *et al.*, 2003; Hülskamp, 2004), cotton fiber cells are used as a model to study plant cell differentiation (Kim and Triplett, 2001; Lee *et al.*, 2007). After an ovular protodermal cell has initiated to become a fiber cell, the

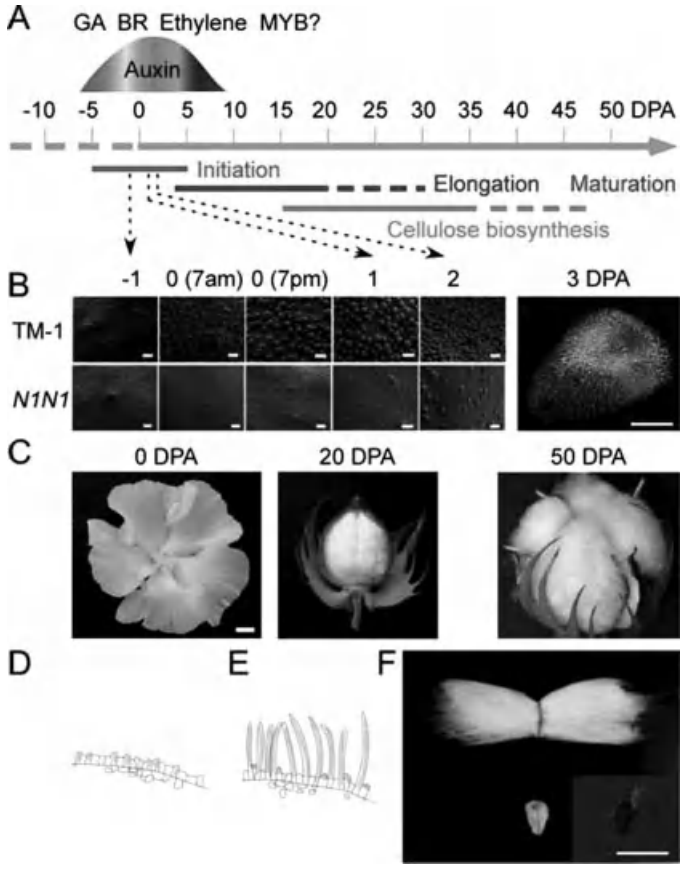

Figure 11.1 Cotton fiber development. *A,* Continuous and overlapping stages of cotton fiber development, including fiber cell initiation, elongation, cellulose (secondary cell wall) biosynthesis, and maturation. During elongation and maturation stages, cellulose biosynthesis is the dominant cellular metabolic process. (Secondary cell wall accumulation starts by depositing the cellulose under the primary cell wall after 15 DPA.) Regulation of phytohormones, including auxin, brassinosteroid (BR), and ethylene and transcription regulators, including MYB proteins, is implicated in cotton fiber cell initiation. *B,* Scanning electron microscope images of cotton fiber initiation processes. TM-1, *G. hirsutum* L. TM-1; N1N1, naked seed mutant. The ovules were collected at −1, 0 (7 a.m. and 7 p.m.), and 1 and 2 days after anthesis (0 = on the day of anthesis). Bar = 20 μm; bar = 100 μm for TM-1 ovule at 3 DPA. *C,* TM-1 cotton flower on 0 DPA, boll on 20 DPA and 50 DPA. *D,* Diagram of cotton fiber lint cell initiation on 0 DPA (green). *E,* Diagram of fuzz fiber cell initiation on 5–7 DPA (orange) along with lint fiber (green). *F*, Mature cotton lint and fuzz fiber on TM-1 seed. *Inset,* Mature seed of the fiberless mutant *XuFL.* (For color detail, see color plate section.)

cell nucleus starts a process of endoreduplication, the cell elongates, and primary and secondary cell walls are formed through biosynthesis of cellulose that can account for >95% of the dry fiber weight after dehydration (Meinert and Delmer, 1977; Kim and Triplett, 2001). An *Arabidopsis* leaf trichome cell undergoes a similar process of nuclear endoreduplication, cell extension, and cell wall biosynthesis (Hülskamp, 2004). The main differences are that leaf trichome development is exposed to light, and the trichome forms 0–3 branches; whereas cotton fiber cell development proceeds in dark conditions under the cover and compact of carpel walls, and the cotton fiber is unbranched.

At the molecular level, cotton seed hair development shares many similarities with *Arabidopsis* leaf trichome development (Lee *et al.*, 2007), which is mediated by a "trichome activation complex"

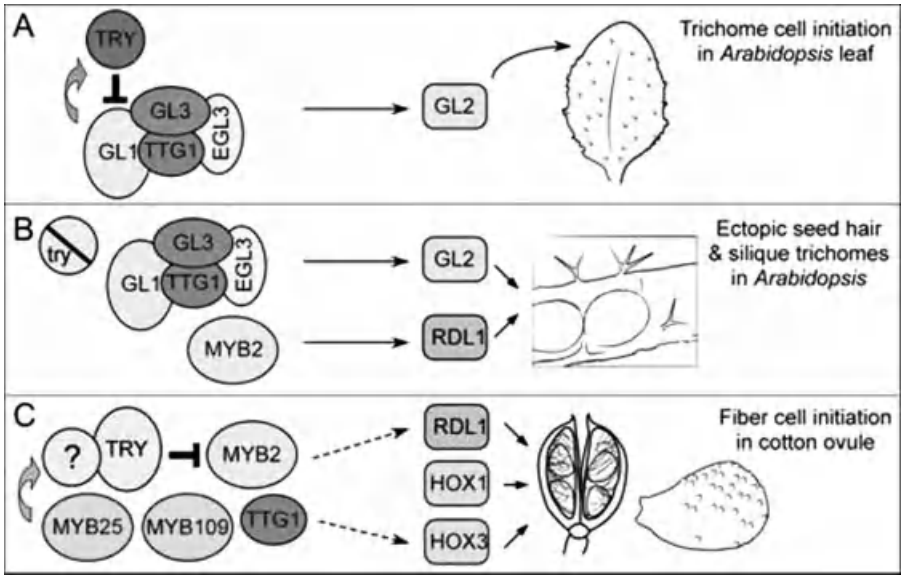

Figure 11.2 Models for leaf trichome and seed hair development. *A,* The trichome initiation complex consisting of GL1, GL3, EGL3, TTG1, and a negative regulator TRY promotes GL2 expression, leading to initiation of leaf trichomes in *Arabidopsis thaliana*. *B,* Cotton fiber genes, including *MYB2* and *RDL1,* stimulate the development of ectopic seed hairs and silique trichomes in *A. thaliana*. *C,* Multiple transcription factors identified in cotton fibers are shown to play roles in the development of seed hair and fiber in cotton. (From Guan *et al.* [2011].) (For color detail, see color plate section.)

(Figure 11.2A) (Szymanski *et al.*, 2000; Esch *et al.*, 2004; Hülskamp, 2004; Ishida *et al.*, 2008; Pesch and Hülskamp, 2009). *Arabidopsis* trichome cell fate is determined by positive transcription regulators, including a R2R3 MYB protein GLABROUS1 (GL1) (Oppenheimer *et al.*, 1991; Larkin *et al.*, 1993; Larkin *et al.*, 1994), a WD repeated protein TTG1 (Walker *et al.*, 1999), and a bHLH protein GL3 (Payne *et al.*, 2000) and its homolog EGL3. These proteins form a complex (Payne *et al.*, 2000; Zhang *et al.*, 2003; Zimmermann *et al.*, 2004) to drive expression of the downstream homeodomain transcription factor gene *GL2* (Szymanski and Marks, 1998). The transcription factor complex also activates another small single repeat R3 MYB transcription factor TRY (Hülskamp *et al.*, 1994; Esch *et al.*, 2004). TRY can competitively disrupt the binding between GL1 and GL3, to function as a feedback negative regulator that suppresses trichome formation (Schellmann *et al.*, 2002; Esch *et al.*, 2003). *Arabidopsis* seeds normally do not develop hair, so little is known about additional genes and regulatory pathways responsible for seed hair formation.

In the cotton genome, all homologs of *Arabidopsis* trichome positive regulators have been identified (Figure 11.2B). *G. hirsutum* MYB109 (GhMYB109) and GhMYB2 are putative MYB transcription factors present in cotton fibers (Suo *et al.*, 2003). Overexpression of *G. arboreum MYB2* (*GaMYB2*) complements a *gl1* mutation (trichomeless) and restores the trichome phenotype in *Arabidopsis thaliana* (Wang *et al.*, 2004). Cotton fiber–related genes *MYB2* and *RDL1* serve as positive regulators for both leaf trichome and seed hair development (Figure 11.2B). In cotton fiber cells, multiple regulators may determine and promote the cell fate for seed hair. The functional homologs of *TTG1*, *GL3*, and *GL2* from the cotton genome are also cloned and studied. Genome-wide analysis of gene expression showed that *G. hirsutum MYB2*, *MYB25*, *MYB109*, *HOX1*, *HOX2*, and *TTG1* are associated with seed hair development (Figure 11.2C). Individual and interactive roles for these

factors in seed hair development are largely unknown. It is likely that multiple factors are required for the initiation of trichomes on reproductive organs such as siliques and hairs on seeds. One gene in *A. thaliana* has multiple homologs in cotton because of its polyploid origin. For example, at least four homologs of *TTG1* (Humphries *et al.*, 2005) and three homologs of *GL2* (Guan *et al.*, 2008) are present in the cotton genome. Searches for homologs of negative regulators have not identified good candidates. These data suggest that *Arabidopsis* and cotton use similar transcription factors for regulating the development of leaf trichomes and seed hairs.

A more recent study has demonstrated that cotton fiber–related genes promote development of seed hair and silique trichomes (Guan *et al.*, 2011). Overexpression of *GhMYB2* and its downstream gene, *GhRDL1*, activated trichome and seed hair pathways in the *try* mutant *A. thaliana*. TRIPTYCHON (TRY) is a R3MYB protein that inhibits trichome formation, and *try* mutants form trichomes in clusters instead of as evenly spaced individuals (Hülskamp *et al.*, 1994; Esch *et al.*, 2004). The transgenic plants developed ectopic trichomes on siliques (Figure 11.3A and B) and

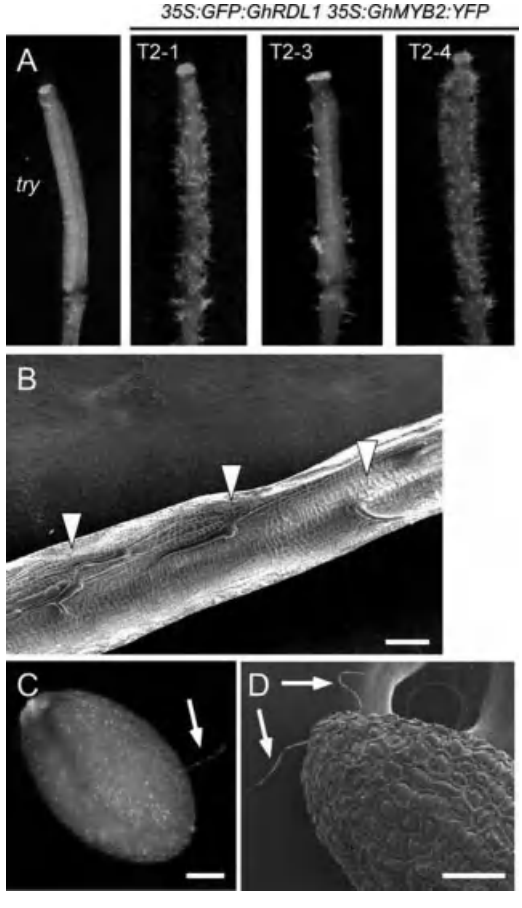

Figure 11.3 Development of ectopic trichomes inside and outside of siliques in transgenic *Arabidopsis thaliana* plants overexpressing *GhMYB2* and *GhRDL1*. *A,* Ectopic trichomes outside of siliques in transgenic *A. thaliana* plants. *B,* Ectopic trichomes inside of siliques *(arrows)* in transgenic *A. thaliana* plants. *C* and *D,* Seed hair observed in transgenic *A. thaliana* seeds under light microscope *(C)* and Scanning electron microscope *(D)*. (From Guan *et al.*, [2011].) (For color detail, see color plate section.)

seed hair in seeds (Figure 11.3C and D), providing strong evidence for cotton fiber–related genes in trichome and seed hair development. Overexpressing *GhMYB2* or *GhRDL1* alone in the *A. thaliana try* mutant that produces multibranched trichomes leads to ~5% of transgenic seeds producing seed hair. Overexpressing both *GhMYB2* and *GhRDL1* results in ectopic trichomes on outer and inner sides of siliques and seed hair development in 10% of the transgenic seeds. GFP:GhRDL1 and GhMYB2:YFP fusion proteins are colocalized in nuclei of ectopic trichomes.

In addition to results in homologs of transcription factor genes in trichome pathways, many cotton genes are found to be highly expressed during fiber development (Wu *et al.*, 2006; An *et al.*, 2007; Chen and Guan, 2011). These genes include *GhMYB25,* which is predominately expressed in ovules and in fiber cell initials (Wu *et al.*, 2006). GhMYB25 is a homolog of AmMIXTA/AmMYBML1 that controls conical cell and trichome differentiation in *Antirrhinum majus* petals (Noda *et al.*, 1994; Perez-Rodriguez *et al.*, 2005). Suppressing *GhMYB25*-like gene expression by RNA interference in cotton impairs fiber development (Walford *et al.*, 2011). *G. barbadense* ML1 (GbML1), a L1 box binding protein present only in cotton, binds GbMYB25 in *G. barbadense*. The data suggest additional and possibly novel cotton genes are required for fiber cell initiation.

Fiber Cell Expansion through Cell Wall Biosynthesis

Cotton fiber cells elongate rapidly shortly after fertilization up to ~20 DPA. During this process, a fiber cell can expand >2 cm in length through primary cell wall expansion and accumulate cellulose for biosynthesis of the secondary cell wall. This elongation process is rapid and associated with elevated expression levels of genes in the functional categories of cell skeleton, carbohydrate metabolism, and expansin (Ruan *et al.*, 2001; Shi *et al.*, 2006; An *et al.*, 2007; Gou *et al.*, 2007; Haigler *et al.*, 2009).

It is well known that functional activities of expansin, xyloglucan, endo-(1,4)-β-D-glucanase, and reactive oxygen species (ROS) are important to the enlargement of primary cell walls (Cosgrove, 2005). Because the primary cell wall expansion determines the cotton fiber cell length, the primary cell wall is critical for the cotton fiber quality. Six cotton *expansin A* genes are expressed differentially in the rapid elongation stage of fiber development (An *et al.*, 2007). High expression levels of cotton *expansin* genes during early stages of cotton fiber development coincide with the active biosynthesis of primary cell wall. Three genes encoding xyloglucan endotransglycosylases/hydrolases (XTH) in cotton are expressed in elongating cotton fiber cells (Lee *et al.*, 2007). *XTH* transcript levels are increased in transgenic cotton plants overexpressing *GhXTH1*, and these plants produce longer fibers (Lee *et al.*, 2010). Two groups of genes – *GhAPXs* encoding ascorbate peroxidase and *GhPOXs* encoding class III peroxidase – are actively expressed during cotton fiber elongation and related to the accumulation of ROS, such as hydroxyl radical (—OH) (Qin *et al.*, 2008; Mei *et al.*, 2009). Genome-wide gene expression analysis found an evolutionary role of ROS in the fiber trait (Hovav *et al.*, 2008). Three genes encoding peroxidases were consistently expressed in domesticated and wild cotton species with long fibers, but expression was not detected in wild species with short fibers. *GhAPX1* and *GhPOX1* are highly induced in cotton fiber cells specifically in the rapid elongation stage (Qin *et al.*, 2008; Mei *et al.*, 2009). ROS functions as wall-loosening agents in living cells. These genes may affect fiber length by participating in primary cell wall extension.

As substrates for biosynthesis of polysaccharides (primary cell wall) and cellulose (secondary cell wall), glucose, fructose, galactose, and sucrose accumulate in rapidly growing cotton fiber cells. At 9 DPA, >90% metabolites in rapidly elongating fiber cells are glucose, fructose, galactose, and sucrose (Gou *et al.*, 2007). This trend of composition is relatively stable in cotton fiber cells from 6–18 DPA. Correspondingly, the genes encoding sucrose and other carbohydrate synthases

and transporters are actively expressed during the fiber elongation stage. The genes that have been investigated encode *G. hirsutum* sucrose transporter1 (GhSUT1) (Ruan *et al.*, 2001), sucrose synthase (GhSUS) (Ruan *et al.*, 2003), vacuolar invertase1 (GhVIN1) (Wang *et al.*, 2010), and β-1,3-glucanase (GhGluc) (Ruan *et al.*, 2004). Sucrose and potassium are major osmotic solutes that are imported into cotton fiber from cells underneath the seed coat. The transport is either through plasmodesmata (PD) or through transporters across the cell wall and plasma membrane, which is facilitated by tonoplast H^+-ATPases (Ruan *et al.*, 2001). *GhKT* encodes a cotton potassium transporter. GhKT and GhSUT1 together may facilitate transport of sucrose and potassium through PD into cotton fiber cells to maintain the turgor pressure that drives the cotton fiber elongation (Ruan *et al.*, 2001, 2004). Sucrose is also one of the substrates for cell wall biosynthesis. In fiber cells, sucrose is degraded into UDP-glucose and fructose by sucrose synthase in the cytoplasm or hydrolyzed by acid invertase into glucose and fructose in the vacuole (Delmer, 1999; Salnikov *et al.*, 2001; Wang *et al.*, 2010). During cellulose biosynthesis, actin and tubulin are uniformly distributed along with sucrose synthases (Salnikov *et al.*, 2003). Transgenic cotton expressing antisense *GhSUS* produces short cotton fibers (Ruan *et al.*, 2003). Overexpressing *GhVIN1* and repressing *GhVIN1* by *ghvin1*-RNAi in transgenic cotton shows positive correlation of *GhVIN1* expression levels with cotton fiber length (Wang *et al.*, 2010). These data suggest that carbohydrate metabolism and transport play roles in fiber cell elongation.

Sucrose is the initial carbon source for cellulose biosynthesis. The cellulose synthase (CESA) uses UDP-glucose as a substrate to produce cellulose (Delmer, 1999). Secondary cell wall deposition begins at 15–18 DPA. Mature cotton fibers consist of >95% of pure cellulose.

Regulation of Phytohormones during Cotton Fiber Development

Cotton fiber development is effected by most plant hormones (Lee *et al.*, 2007). Auxin and GA are known to affect fiber cell development (Beasley, 1973). Under *in vitro* culture conditions, a combination of 500 nM auxin [indole-3-acetic acid (IAA)] with 5.0 mM GA is found to be optimal for fiber production (Beasley and Ting, 1974). Ethylene and brassinosteroid (BA) can also positively affect fiber growth in cultured ovules at certain concentrations (Sun *et al.*, 2005; Shi *et al.*, 2006; Clark *et al.*, 2010). Microarray analysis of gene expression in elongating cotton fibers identified an enrichment of genes in ethylene biosynthesis and signaling pathways (Shi *et al.*, 2006). Further analysis found a role for ethylene in fiber cell elongation. In cotton, ethylene affects auxin biosynthesis and transport (Beyer and Morgan, 1969), and ethylene function is affected by GA activity (Morgan and Durham, 1975). It is likely there are interactive roles for different growth hormones in cotton fiber cell initiation and development.

The mechanisms for how phytohormones stimulate cotton fiber development are poorly understood. Genome-wide analysis of expressed sequence tags (ESTs) and transcriptomes suggests that some genes involved in phytohormone biosynthetic and signaling pathways are highly expressed at different stages of cotton fiber development. In cotton fiber EST collections, the enriched genes include homologs of GA biosynthetic genes—*GA20ox, GA3ox,* and *KO*—and genes involved in GA signaling pathways, such as *GAI, RGL2, RGL1, DDF1, PHOR1, RSG, POTH1*, *PKL, GL1, GAMYB, AGAMOUS*, and *LUE1* (Yang *et al.*, 2006). DELLA protein, an important GA signaling transduction signal component (Richards *et al.*, 2001), also accumulates during early stages of cotton fiber development (Guan *et al.*, 2011).

A list of phytohormone-related genes identified from two microarray experiments is shown in Table 11.1. These genes were expressed at high levels in ovules (−2 DPA) and fibers (3–20 DPA). Although the function of individual genes in cotton fiber development has yet to be elucidated, this

Table 11.1 Alternative Expressed Phytohormone-Related Genes in Cotton Fiber

Phytohormone	Gene	Description	Reference
GA	*GA20ox1*	Gibberellin 20-oxidase 1	1
	GA3ox1	Gibberellin 3-hydroxylase 1 mRNA	1
	DELLA	Negative regulators of gibberellin acid (GA) *signaling*	2
Auxin	*ARF2*	Auxin response factor 2	2
	ARF6	Auxin response factor 6	2
	ARF11	Auxin response factor 11	2
Ethylene	*RTE1*	Reversion-to-ethylene sensitivity1	2
	ERF3	Ethylene responsive element binding factor 3	2
	ERF4	Ethylene responsive element binding factor 4	2
	EIN2	Ethylene insensitive 2	2
	ACO1	The 1-aminocyclopropane-1-carboxylic acid oxidase 1	1
	ACO2	The 1-aminocyclopropane-1-carboxylic acid oxidase 2	1
	ACO3	The 1-aminocyclopropane-1-carboxylic acid oxidase 3	1
BR	*BRI1*	Brassinosteroid insensitive 1	2
	SMT1	24-sterol C-methyltransferase (SMT1)	1
	DET2	Steroid 5-alpha-reductase	1
ABA	*ABI5*	ABA insensitive5	2

[1]2 Shi *et al.*, 2006.
[2]Guan *et al.*, 2011.

group of phytohormone genes whose expression is enriched in fiber tissues suggests complex and interactive roles for these major phytohormones in cotton fiber cell development.

Auxin response factor (ARF) family gene transcripts are enriched in cotton EST collections and microarray analysis (Yang *et al.*, 2006; Lee *et al.*, 2007; Guan *et al.*, 2011). *ARF2*, *ARF6*, and *ARF11* are actively expressed in the cotton fiber cells in the early development stage (Guan *et al.*, 2011). Synthetic NAA suppresses secondary cell cellulose synthesis but increases cell elongation (Singh *et al.*, 2009). A study employed different promoters to drive expression of the IAA biosynthetic gene *iaaM* and control endogenous auxin in cotton ovules (Zhang *et al.*, 2011). The promoters that are able to increase IAA from −2 to 0 DPA in the ovule epidermal layers result in cotton plants that produce more and longer fiber cells and finer fibers. This suggests both timing and location of auxin production are important for cotton fiber production (Figure 11.1A).

Cotton Fiber Genes in Diploid and Tetraploid Cotton

The genus *Gossypium* includes ∼45 diploid (2n = 2x = 26) and five tetraploid (2n = 4x = 52) species (Percival *et al.*, 1999). Diploid species fall into eight genomic groups (A–G and K). The African clade (A, B, E, and F genomes) originated in Africa and Asia, whereas the D-genome clade is indigenous to the Americas. A third diploid clade (C, G, and K genomes) is found in Australia. The most widely cultivated cotton is tetraploid. Polyploidization is estimated to have occurred 1–2 million years ago (Mya) (Wendel and Cronn, 2003), giving rise to five extant allotetraploid species. Two cultivated species, *G. hirsutum* L. and *G. barbadense* L., are classic natural allotetraploids that arose in the New World from interspecific hybridization between an A-genome–like ancestral African species and a D-genome–like American species. The closest extant relatives of the original tetraploid progenitors are the A-genome species *G. herbaceum* L. (A1) and *G. arboreum* L. (A2) and the D-genome species *G. raimondii* (D5) Ulbrich (Brubaker *et al.*, 1999). The A-genome species

produce spinnable fiber and are cultivated on limited scale, whereas the D-genome species do not (Applequist *et al.*, 2001). Both the A-subgenome and the D-subgenome in the allotetraploids contribute to superior fiber traits (Jiang *et al.*, 1998; Saha *et al.*, 2006; Yang *et al.*, 2006). The fiber in allotetraploids is much longer and stronger compared with that in diploids, suggesting activation or silencing of homoeologous fiber-related genes via genetic and epigenetic mechanisms (Wendel and Cronn, 2003; Adams and Wendel, 2005; Chen, 2007; Lee *et al.*, 2007).

The genomic interactions in allotetraploid cotton are predicted to affect gene expression related to superior fiber quality. The existing data are debatable. Based on the distribution of fiber-related quantitative trait loci (QTLs), some studies indicate that contributions of the D subgenome are more significant to lint fiber development than the contributions of the A subgenome in allotetraploid cotton (Jiang *et al.*, 1998; Rong *et al.*, 2007), although the D genome ancestor does not produce fiber. However, other studies suggest that the A subgenome is more important than D subgenome for the fiber trait based on analysis of QTLs, EST collections, and gene expression (Mei *et al.*, 2004; Ulloa *et al.*, 2005; Yang *et al.*, 2006). Additional results argue that the two subgenomes are equally important for fiber development (Han *et al.*, 2006; Xu *et al.*, 2010). These discrepancies are probably caused by the lack of cotton genome sequence and partially informed knowledge about fiber gene expression. Distribution of QTLs in the cotton genome is uneven, and many QTLs are clustered in some regions of chromosomes (Mei *et al.*, 2004; Ulloa *et al.*, 2005; Rong *et al.*, 2007; Wang *et al.*, 2007). QTL clusters for fiber yield and quality may suggest multiple *cis*-acting and *trans*-acting factors that affect cotton fiber cell growth and development.

Further genetic improvement of cotton fiber production greatly depends on genome sequences. The strategy for sequencing cotton genomes has been outlined by the cotton community (Chen *et al.*, 2007). Toward that goal, physical maps of two diploid progenitors, *G. arboreum* and *G. raimondii*, and the most widely cultivated allotetraploid cotton, *G. hirsutum*, have been developed. *G. raimondii* has been prioritized for complete genome sequencing in collaboration with the Department of Energy (DOE) Joint Genome Institute (Chen *et al.*, 2007; Lin *et al.*, 2010). This physical map consists of 13,662 BAC-end sequences and 1585 contigs that are anchored to a cotton consensus linkage map with 2828 probes. The Gossypium raimondii genome was just seqeunced (Wang *et al.* 2012), and another version is expected to be made available soon. The construction of several BAC libraries and physical maps of the A-genome species *G. arboreum* and the allotetraploid species *G. hirsutum* are also underway (Ruan *et al.*, 2004). These physical maps will provide frameworks for sequence assembly and ultimate sequencing of allotetraploid cotton genomes.

Roles for Small RNAs in Cotton Fiber Development

Small RNAs, including small interfering RNAs (siRNAs), microRNAs (miRNAs), and trans-acting siRNAs (ta-siRNAs), affect gene expression and epigenetic regulation in plants and animals (Baulcombe, 2004; Chapman and Carrington, 2007; Chen, 2009). Many transcription factor and phytohormone regulator genes such as *ARFs* are regulated by miRNAs (Mallory *et al.*, 2005; Gutierrez *et al.*, 2009), and these target genes are actively expressed in cotton fibers (Guan *et al.*, 2011), suggesting a role for small RNAs in cotton fiber development.

Small RNAs have been investigated in cotton ovules at 0–10 DPA, and 583 unique sequences were identified (Abdurakhmonov *et al.*, 2008). Most of these small RNA sequences (62.1%) are 24-nt long. Using *Gossypium* EST databases, further analysis found 22 conserved miRNA precursors, including 15 from *G. hirsutum* (AADD), 5 from *G. raimondii* (DD), and 1 from *G. herbaceum* (AA) and *G. arboreum* (AA) (Khan Barozai *et al.*, 2008). A comparative analysis of small RNA expression

changes between the wild-type and fiberless (*fl*) mutant in ovules at 0–10 DPA revealed 22 conserved candidate miRNA families including 111 members (Devor *et al.*, 2009). Some miRNA families display differential expression in ovules between the wild-type and *fl* mutant (Kwak *et al.*, 2009).

High-throughput sequencing analysis of small RNAs revealed differential expression of siRNAs and miRNA in fiber-bearing tissues (cotton ovules at −3 through 3 DPA cotton ovule) and nonfiber tissues (leaves) (Pang *et al.*, 2009). During early stages of cotton fiber development, 24-nt siRNAs are highly enriched, and 21-nt miRNAs are severely repressed. The enrichment of 24-nt siRNAs (78%–84% of total small RNA reads in fiber-bearing ovules) and relative to leaves suggests a role for these siRNAs that are yet to be uncovered in fiber cell development. Most of these 24-nt siRNAs are derived from transposable elements (TEs) and TE-associated genes. TEs are associated with gene silencing and RNA-directed DNA methylation (RdDM) (Mette *et al.*, 2000; Law and Jacobsen, 2010). Although the origin of these 24-nt small RNAs is unknown owing to the lack of the cotton genome sequence, the enrichment of 24-nt small RNAs in cotton ovules and fibers is reminiscent of maternal inheritance of a major class of 24-nt siRNAs whose biogenesis is dependent on RNA polymerase IV (PolIV or p4) (or p4-siRNAs) in developing seeds (Mosher *et al.*, 2009). Rapid and dynamic changes in siRNA and miRNA expression in cotton fibers are reminiscent of changes observed in *Arabidopsis* allotetraploids and their progenitors (Ha *et al.*, 2009). Cotton fibers are derived from maternal tissues in the epidermal layer of seeds. Enrichment of 24-nt siRNA in cotton ovules may suggest roles for these siRNAs in RdDM and gene regulation during cotton ovule development, fiber differentiation, and rapid growth and regulation of homoeologous genes in the allotetraploid genome.

Repression of miRNAs in cotton ovules and fibers is associated with upregulation of many genes encoding transcription factors and phytohormone regulators (Pang *et al.*, 2009). Target genes have been identified for >30 families of miRNAs, and transcript cleavage events have been documented for some miRNAs. These miRNAs cleave target gene transcripts that encode transcription factors, such as homeodomain (HD) proteins, APETAL2 protein, and phytohormone response factors such as ARFs. These results suggest that miRNAs play a role for regulating expression of these transcription factor genes in cotton fiber cell development. One of the new identified cotton miRNAs, Gh-miR2948, targets a gene encoding a sucrose synthase-like protein, suggesting a novel role for this miRNA in carbohydrate metabolism in cotton fibers. Although several new miRNAs and miRNA targets have also been identified, understanding the genome-wide landscape of regulatory pathways that are programmed by siRNAs and miRNAs during fiber cell development requires the cotton genome sequence, which is predicted to be available in the near future (Chen *et al.*, 2007; Lin *et al.*, 2010).

Conclusion

Economically, cotton is the largest source of renewable fibers for the textile and home furnishing industries, and cottonseed oil provides unique features of flavor and stability in cooking and food preparation. Biologically, cotton fiber is a model system to study plant cell differentiation, expansion, and wall biosynthesis. Analysis of cotton fibers is leading to a significant translational genomic interface with leaf trichomes, seed fibers, xylem elements, and other cells with thick walls. The cotton genus *Gossypium* is also a model for study of plant genome evolution, including polyploidization. With anticipated completion of genome sequences in diploid and allotetraploid cotton, genomic research in cotton will reveal new knowledge and information with respect to cell fate determination, cell elongation, cellulose biosynthesis, and polyploidization. Roles for phytohormone regulation and

small RNAs in cotton cell differentiation and elongation are expected to generate broader impacts in the basic understanding of cell biology and small RNA biology as well as in the improvement of cotton quality and yield through application of genome-enabled biotechnological tools.

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12 Genomic Changes in Response to 110 Cycles of Selection for Seed Protein and Oil Concentration in Maize

Christine J. Lucas, Han Zhao, Martha Schneerman, and Stephen P. Moose

Introduction

Identification of the genetic mechanisms underlying phenotypic variation between and within populations is essential to understanding natural selection and evolution. Changes in gene expression are an important source of phenotypic variation and may arise as a result of a combination of *cis*-regulatory and *trans*-regulatory variation within complex genetic networks (Wittkopp, 2005). This complexity has hindered identification of genomic targets of both natural and artificial selection. Long-term phenotypic selection experiments in plants and animals provide an invaluable resource for studying evolutionary change and regulatory variation, with implications for enhancing the efficiency of genetic gain in plant and animal breeding (Dudley and Lambert, 2004). Plants have the additional unique advantage of regeneration, where replicate selection experiments may be conducted in parallel. The Illinois Long-Term Selection Experiment for grain composition is a classic in plant genetics; >800 cycles of selection have been conducted from the same source population during the past century (Figure 12.1).

Previous quantitative trait loci (QTL) mapping studies suggest that changes in both protein and oil concentration in the Illinois experiment are governed by many genes (Laurie *et al.*, 2004; Dudley *et al.*, 2007) with small phenotypic effect. However, there is also evidence that variation at relatively few loci can be associated with significant phenotypic differences. Advances in high-density genotyping coupled with functional genomics approaches enable a more thorough understanding of the genetic basis for the dramatic phenotypic responses observed. In this chapter, we provide an update on more recent phenotypic responses within the Illinois Long-Term Selection Experiment and related populations, a review of past efforts to characterize the genetic architecture of selection response, and a preliminary view of gene expression changes resulting from divergent selection for grain protein, and we introduce the use of promoter-reporter genes as tools to understand how selection has altered the regulation of seed storage protein genes. Collectively, these approaches illustrate that long-term selection for kernel composition has targeted variation in both trans-acting regulatory factors and specific genes known to influence oil or protein concentrations.

Background on the Illinois Long-Term Selection Experiment

The Illinois Long-Term Selection Experiment was initiated in 1896 at the University of Illinois by Cyril G. Hopkins and was the first planned attempt to improve cereal grain quality through

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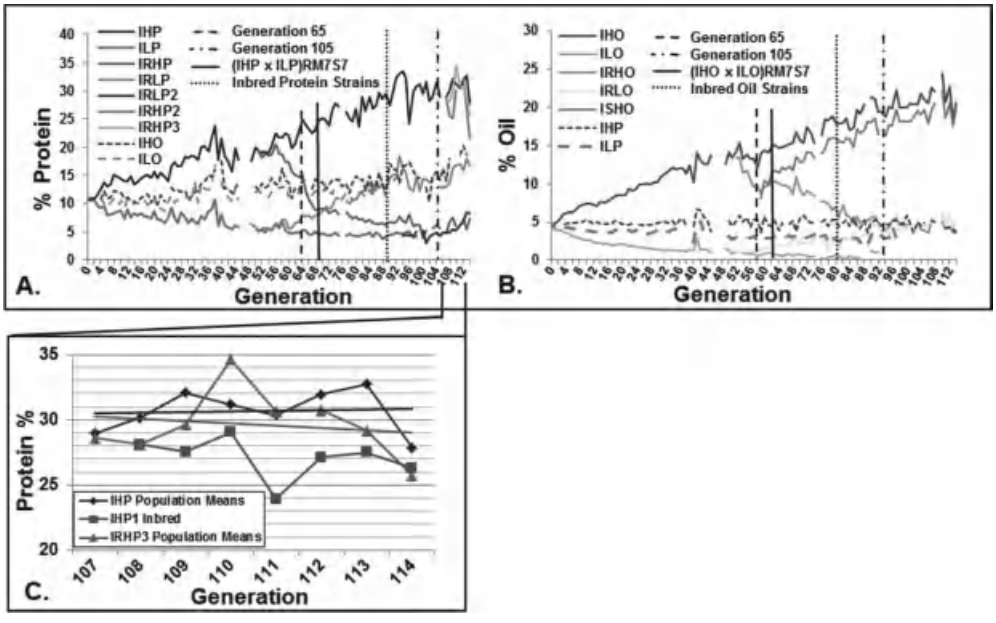

Figure 12.1 *A* and *B,* Selection responses in the Illinois Protein Strains *(A)* and Illinois Oil Strains *(B)*. Protein concentrations for IHO and ILO are plotted as *dashed lines* in *(A)*, and oil concentrations for IHP and ILP are plotted as *dashed lines* in *(B)*. The vertical lines highlight important generations of the experiment: generation 70, from which QTL mapping populations have been derived from crosses of selected strains; generation 90, as a source of inbred lines derived from either the Illinois Protein or Oil Strains; generation 65, the oldest for which seed is still available; and generation 105, a recent generation used for comparison to generation 65. *C,* Expanded view of the last eight cycles of selection response in IHP and Illinois Reverse High Protein 3 compared with IHP1 inbred line. (For color detail, see color plate section.)

breeding (Moose *et al.*, 2004). It took <10 generations to do so (Smith, 1908). The objective was later modified to determine the limits of selection for grain protein and oil concentrations in maize, and the experiment has since undergone 114 generations of recurrent selection, making it the longest running continuous genetics experiment in higher plants. Briefly, the experiment began by measuring grain protein and oil concentrations from 163 ears of the open-pollinated maize variety, "Burr's White." The 24 ears with the highest protein concentrations were selected as the parents for Illinois High Protein (IHP), the 24 with the highest oil concentrations for Illinois High Oil (IHO), and the 12 ears lowest for these traits became parents of Illinois Low Protein (ILP) and Illinois Low Oil (ILO) (Hopkins, 1899). Although the breeding scheme has varied throughout the experiment, the methods employed were chosen to minimize inbreeding. During the past 40 cycles, selected ears from each strain were divided into two groups, with controlled pollinations performed only between groups and using each individual plant as a parent only once to generate at least 60 ears from each strain for analysis. Similar cycles of selection have been conducted annually to the present except for during World War II. The resulting four populations span the known extremes for grain protein and oil compositions; compared with an initial population mean of 11% grain protein and a range of 8%–14%, IHP kernels contain >32% protein and ILP kernels only 4% protein (Figure 12.1*A*). Similarly, compared with an initial population mean of 5% grain oil and a range of 3%–5%, IHO kernels contain >20% oil, and ILO kernels contain 1% oil (Figure 12.1*B*). Additional details about selection procedures throughout the course of the experiment can be found in Dudley and Lambert

(2004), and the historical impacts of the experiment to the fields of plant breeding and genetics can be found in Moose *et al.* (2004).

To determine the extent of genetic variability and phenotypic response remaining after 48 generations of forward selection, the direction of selection in the IHP, ILP, IHO, and ILO populations was reversed using the same method of selection as the forward strains (Woodworth *et al.*, 1952). Low protein ears were selected from IHP to create Illinois Reverse High Protein (IRHP), and high protein ears were selected from ILP to create Illinois Reverse Low Protein (IRLP) (Figure 12.1*A*). Illinois Reverse High Oil (IRHO) and Illinois Reverse Low Oil (IRLO) were created in the same way from IHO and ILO (Figure 12.1*B*). Illinois Switchback High Oil (ISHO) was initiated from cycle 55 of IRLO with the same overall goal of determining genetic variation. The response to selection began to plateau in ILP by cycle 60, but selection was continued until cycle 93, when the strain was discontinued because of poor germination and lack of phenotypic response. To determine the level of genetic variation remaining in ILP, Illinois Reverse Low Protein 2 (IRLP2) selection was initiated from cycle 90. The ILO selections were also discontinued because of a lack of method for detecting such small differences in oil ($<$1%). Two new reverse selections are introduced here, Illinois Reverse High Protein 2 and 3 (IRHP2 and IRHP3), which were initiated from cycle 103 of IHP by selecting for low protein individuals. They were created for three reasons: (1) to estimate the extent of genetic variability remaining in IHP after $>$100 cycles of forward selection, (2) to assess the impact of soil N fertility on phenotypic means and selection response, and (3) to initiate new replicated populations where DNA and seeds of the founding individuals are preserved for future analysis of genetic responses to selection.

In addition to being analyzed for their respective traits, grain protein concentrations were measured from the bulks of IHO and ILO ears (Figure 12.1*A*). Similarly, grain oil concentrations were measured from the bulks of IHP and ILP ears (Figure 12.1*B*). The relatively limited change in these phenotypes throughout the experiment confirms that oil and protein concentrations are not significantly correlated, and IHP and ILP serve as unselected controls for the oil strains. Likewise, IHO and ILO serve as unselected controls for the protein strains. The unselected controls provide an estimate of the impacts of environmental variability and the extent of genetic drift in these populations. The mean protein concentrations for IHO and ILO show a slight positive trend since 1896 and range from 8%–20% protein throughout the experiment. The oil means of IHP and ILP have varied only by 2%–7% oil. The plots also reveal that throughout the selection experiment, protein is more sensitive to environmental fluctuations than oil. For example, mean protein concentrations fluctuated widely during generations 90–98, where 1993 conditions appeared to be a highly favorable for protein deposition but 1995 conditions were very poor.

Phenotypic Responses to Selection

The phenotypic responses to artificial selection of the Illinois Protein and Oil Strains can be described as steady and prolonged, with the exception of IRHP. This observation suggests a complex genetic architecture and high levels of genetic variation. The sustained progress and continuous phenotypic response observed in these strains is characteristic of quantitative traits controlled by many genes and consistent with the results of genetic mapping studies using progenies created by the cross of IHP x ILP and IHO x ILO. Estimates of genetic variability and genetic gain based on quantitative genetics theory have been reported at various times throughout the experiment and are reviewed by Dudley *et al.* (2007). IHP, IHO, and SHO still exhibited significant realized heritability, and IHO and ISHO also exhibited significant change per generation. An attempt was made to assess genetic

variability of ILP via genetic response in IRLP2, which was initiated three cycles before the last cycle of selection in ILP. However, these results were inconclusive.

Accounting for environmental effects becomes more important as phenotypic responses to selection begin to plateau, and the magnitude of phenotypic differences owing to environmental factors becomes relatively large compared with genetic response. A new approach to assess genetic gain is to normalize changes in population means to values measured in homozygous inbred lines derived from the selected populations. For the Illinois Protein Strains, inbred lines derived from generation 90 of the Illinois Protein Strains (named IHP1, ILP1, IRHP1, and IRLP1) and described in Uribelarrea *et al.* (2004) can be used. Each of these inbred lines has been grown on a yearly basis since 2004 (generation 104) in the same field plots, and kernel composition has been estimated by near infrared reflectance (NIR). Because they are genetically fixed inbreds, observed phenotypic differences can be attributed to environmental or possibly epigenetic variation. Figure 12.1*C* illustrates differences in protein concentration between the IHP1 inbred line and the IHP and IRHP3 populations since generation 104. Except for generation 111, the protein concentrations for the IHP1 inbred are consistently between 28% and 30%. The IHP population mean continues to exhibit a slight positive trend and is always higher than IHP1. Conversely, IRHP3 appears to exhibit slightly lower concentrations than IHP in the last three cycles. Inbred lines from the most recent cycles of the Illinois Oil Strains are also being generated, as indicated by the vertical dotted lines in Figure 12.1.

Phenotypic selection will continue in the Illinois Protein and Oil Strains until a limit to selection is definitively observed. As genomics tools become increasingly available and QTL identified for these traits, it would be interesting to determine if genetic gain can be enhanced in these populations or perhaps revived in ILP using marker assisted selection.

Additional Traits Affected by Selection

Numerous additional traits have been affected by selection in the Illinois experiment. It was quickly observed that selection for grain protein concentration operated indirectly on other traits (Hopkins *et al.*, 1903; Smith, 1908). Grain starch concentration and kernel size and hence grain yield were each shown to be inversely related to grain protein concentration in all of the Illinois Protein Strains (Below *et al.*, 2004). Selection for high protein has also resulted in greater lodging and shorter plant height and increases in successful germination and tillering (Woodworth *et al.*, 1952). These traits are oppositely affected by selection for low protein, where the poor germination of ILP was one of the reasons it was discontinued.

Whole-plant nitrogen metabolism may also be altered by selection for grain protein concentration. The nitrogen assimilated by the plant during vegetative development accumulates and is stored as kernel protein until needed as a source of nitrogen by the developing seedling. Physiological changes affecting nitrogen metabolism in response to selection for grain protein concentration were initially noted by Hoener and DeTurk (1938) and have been reviewed by Below *et al.* (2004). The results of these studies demonstrate elevated nitrogen uptake, nitrogen assimilation by seedling leaves, and nitrogen remobilization from source to seed sink tissues of IHP compared with ILP. Uribellarrea *et al.* (2004, 2007) also found that hybrids derived from the Illinois Protein Strains exhibited differences in agronomic nitrogen use efficiency. IHP was also more efficient at remobilizing nitrogen than IRLP. One underlying cause of these physiological differences is changes in the activities of the enzymes involved in these pathways (Dembinski *et al.*, 1991; Below *et al.*, 2004). Because of the inverse relationship between protein and starch, activities of key carbon and metabolism

and starch biosynthesis enzymes have also been examined for response to selection, including ADP-glucose pyrophosphorylase, that exhibits enhanced activity in ILP (Reggiani *et al.*, 1985; Below *et al.*, 2004).

Finally, increased soil nitrogen availability, owing to use of fertilizers beginning with generation 53, has been proposed as one environmental factor that has contributed to continued increases in grain protein concentrations within IHP (Dudley and Lambert, 2004). Cereal seed protein concentrations are known to increase after nitrogen fertilization, and this has been observed in hybrids derived from the Illinois Protein Strains (Uribelarrea *et al.*, 2004). One reason for creating IRHP2 and IRHP3 was to test directly the effect of nitrogen fertilizer application on protein concentration. These strains are derived from IHP, which should be most sensitive to soil nitrogen levels. IRHP2 plants were grown for 3 consecutive years (2004–2006) without supplemental nitrogen in a nitrogen-responsive field, and IRHP3 plants were grown in adjacent plots supplemented with 200 kg N per hectare. The protein concentrations of the IRHP2 population were 2.6%–4.5% lower than IRHP3 over the 3 years. Therefore, although N fertilizer may account for some of the recent increase in grain protein concentration, it does not fully explain the continued phenotypic response of IHP throughout the experiment.

Unlimited Genetic Variation?

The continuous phenotypic response to selection in the Illinois Selection Strains may result from existing genetic variation or arise from novel mutations and recombination. It can occur at the DNA, RNA, protein, or epigenetic levels. Here we review estimates of genetic variation in the Protein and Oil Strains and discuss possible reasons why genetic variation has been maintained despite intense phenotypic selection. One inherent source of genetic variation in quantitative traits arises owing to an extensive number of genes and alleles regulating the trait, where selection has not yet fixed all alleles contributing favorably to the phenotype. This is a plausible scenario in the Illinois experiment because of the following measures taken to minimize inbreeding and retain variation: first, a large number (163) of individuals were used to create the original population; second, the strains are derived from an open-pollinated variety, Burr's White; third, a mating design that minimizes inbreeding has been employed throughout the experiment. Furthermore, the maize genome exhibits a high level of both nucleotide and structural genetic diversity (Chia *et al.*, 2012). Maize also exhibits a low level of linkage disequilibrium (LD), where estimated average decay distances ranging from 1–10 kb have been reported among maize populations.

It is also likely that selection has targeted different genes throughout the course of the experiment. Although fixation of all favorable alleles is unlikely for the above-described reasons, the genes contributing the largest proportion of phenotypic variance or with high allele frequencies are more likely to become fixed, exhausting at least some initial variation. For sustained gains, additional targets with smaller effects or low allele frequencies may become increasingly important. Selection may also act on novel genetic variation arising from spontaneous mutation and recombination. However, as discussed by Laurie *et al.* (2004), responses due to new mutation are typically accompanied by drastic changes in phenotype, a pattern inconsistent with the observations of steady gain in the Illinois Protein and Oil Strains. The rapid decline in IRHP may be an exception to this observation and merits further discussion (see later). Structural variation is yet another possibility, where unequal crossing over during meiosis could generate novel alleles. Intraspecific structural variation has been observed in maize using cytogenetic and flow cytometric approaches, but sequence-based

approaches allow for higher resolution studies. Whole-genome, array-based, comparative genomic hybridization (CGH) is one approach for studying structural variation at the genome scale and has become possible with the sequencing of the B73 genome. A high rate of copy number variation (CNV) and presence/absence variation (PAV) was observed between maize inbred lines B73 and Mo17 (Springer *et al.*, 2009). CGH could be used to investigate genome-wide structural variation between the Illinois Selection Strains.

Selection can also act on epigenetic regulatory variation. Three major mechanisms for initiation and maintenance of epigenetic silencing known to occur in plants are DNA methylation, histone modification, and RNA-associated silencing (Egger *et al.*, 2004). DNA methylation is one underlying mechanism of genomic imprinting, a type of epigenetic modification causing differential expression of a gene depending on the sex of the parent that transmits it. Two types of genomic imprinting exist in plants: allele imprinting, where only alleles from a certain genetic background are affected by parent-of-origin-specific gene expression, and locus imprinting, where all known alleles from different backgrounds are under parent-of-origin control (Gehring *et al.*, 2004) (see Chapter 4). The α-zein genes in maize are known to be regulated by maternal imprinting (Chaudhuri and Messing, 1994), which in plants occurs primarily in the endosperm tissue (Gehring *et al.*, 2004). It has been hypothesized that the evolutionary role of maternal imprinting in the endosperm might be to maintain control of gene expression affecting kernel growth and development because of the dependency of kernel development on the maternal organ, the cob (Alleman and Doctor, 2000). Maternal imprinting of the α-zein genes occurs via allele imprinting, where the zeins exhibit differential methylation patterns depending on their parental heritage. It is proposed that methylation of paternal alleles inhibits zein gene expression by altering the chromatin structure of certain zein genes, whereas maternal alleles are demethylated and derepressed (Lund *et al.*, 1995; Lauria *et al.*, 2004); for review, see Bird and Wolffe, 1999. Parental imprinting is also known to regulate expression of an allele of the *dzr1* locus, a posttranscriptional regulator of the 10-kDa δ-zeins (Chaudhuri and Messing, 1994). Additional studies are required to determine if epigenetic variation has contributed to the phenotypic variation observed in the Illinois Selection Strains, and testing for differential methylation of the zein genes is a good starting point. RNA-associated silencing is also being investigated via RNASeq as a potential mechanism of epigenetic regulatory variation of the Selection Strains.

Genetic Response to Selection: QTL Mapping in the Crosses of IHP x ILP and IHO x ILO

It is apparent from previous research that long-term selection for grain protein concentration has altered numerous related traits, and from this it may be hypothesized that many genes have also been affected (Moose *et al.*, 2004). However, the number and identity of genes remain unknown. Genomics approaches will be increasingly important for understanding the genetic and molecular changes that have altered the genomes of the Illinois Selection Strains as a result of long-term artificial selection. QTL mapping is one approach for studying the genetic architecture of quantitative traits. Several QTL mapping studies have been conducted using populations created by crossing individuals from cycle 70 of IHP and ILP or IHO and ILO (Dudley, 1977) and are reviewed by Dudley *et al.* (2007). QTL influencing protein (Goldman *et al.*, 1993, 1994; Dijkhuizen *et al.*, 1998; Sene *et al.*, 2001; Dudley *et al.*, 2004) and oil (Laurie *et al.*, 2004) have been identified. The results of these studies suggest the presence of many QTLs having small phenotypic additive effects, which is consistent with theoretical estimates for the number of effective genetic factors based on genetic variances, 102–178 genetic factors for protein and 14–69 factors for oil (Moose *et al.*, 2004).

One QTL mapping study using a cross of IHO (cycle 90) and a derivative of ILO that had also been selected for early maturity identified a major-effect QTL on chromosome 6 associated with the ratio of oleic to linoleic fatty acid content QTL that was linked to a region containing the *linoleic acid 1* (*lnl1*) locus (Alrefai *et al.*, 1995). Another study also identified a major-effect QTL on chromosome 6 that accounted for 11% and 9.5% of the variation associated with maize seed oil content and embryo oil concentration (Zheng *et al.*, 2008). The mapping population used in this latter study was derived from the cross of a high-oil inbred line, ASKC28IB1, where IHO was 1 of 56 varieties included in the base population of ASK (Lambert *et al.*, 2004), and a normal-oil inbred line, PH09B. Fine-mapping revealed the presence of a gene encoding an acyl-CoA:diacylglycerol acyltransferase (DGAT1-2), an enzyme that catalyzes the final step in oil synthesis. The ASKC28IB1 allele of DGAT1-2 was shown to contain several polymorphisms, including a phenylalanine insertion at position 469, which was responsible for the increased oil content and embryo oil concentration. Because higher oleic acid content was also associated with near-isogenic lines homozygous for the ASKC28IB1 allele, the DGAT1-2 region was sequenced in IHO. The results indicate that the *ln1* locus in the previous study and the ASKC28IB1 allele of DGAT1-2 are one and the same. It was also shown that although ancestral varieties of maize, including 46 accessions of teosinte lines, contain the ASKC28IB1 allele, most modern inbred varieties of maize, including B73 and Mo17, contain the PH09B allele. Introgression of this DGAT1-2 allele increased kernel oil concentrations in the inbred parents of a current elite Chinese hybrid (Chia *et al.*, 2012).

Identification of factors regulating protein has been more difficult, but strategies for increasing the precision of marker-QTL associations have been attempted. Strong selection in artificial selection experiments is known to increase linkage disequilibrium (LD), which can reduce the precision of associations between markers and QTL and between QTL. Random mating within a mapping population is a strategy for reducing LD through recombination and the breaking up of linkages (Dudley, 1994). Owing to the intensity of selection in the Illinois Selection Strains, random mating of the progeny of the cross of IHP x ILP and IHO x ILO was a strategy employed to reduce LD and break up coupling-phase linkages among genes controlling these traits. Studies by Dudley *et al.* (2004) for IHP x ILP and Willmot *et al.* (2006) for IHO x ILO showed large reductions in the percentage of markers declared significant between the Syn0 (one generation of random mating the F1) and Syn4 (four generations of random mating the F1) populations. These studies revealed the need for a larger number of markers with which to associate the increased number of linkage blocks incurred as a result of recombination.

Subsequent mapping studies employed ~500 SNP markers genotyped on populations following either 7 (IHP x ILP) (Dudley *et al.*, 2007) or 10 generations of random mating (IHO x ILO) (Laurie *et al.*, 2004; Clark *et al.*, 2006). However, given the high frequency of recombination events and reductions in LD within these random-mated populations, 500 markers was likely insufficient to detect with high probability most marker-trait associations. In addition, these more recent genetic mapping studies are complicated by population structure owing to the use of multiple parents from the respective selected populations and the phenotyping of segregating families rather than inbred lines. Despite these past efforts to map genomic regions regulating protein and oil, the number and identity of genes influencing these traits remain largely unknown.

New Mapping Population: Illinois Protein Strain Recombinant Inbreds

To overcome the limitations of the previously described mapping populations, a new recombinant inbred mapping population was created: the Illinois Protein Strain Recombinant Inbred (IPSRI)

population. The IPSRI population consists of 500 recombinant inbred lines derived from the cross of cycle 70 IHP and ILP plants, followed by seven generations of random mating (Dudley *et al.*, 2007) and six generations of inbreeding (Moose laboratory). This population exhibits a normal phenotypic distribution, ranging from 5%–24% protein, and captures most phenotypic variation in the IHP and ILP populations at cycle 70. It also consists of a large number of individuals, which should provide adequate power to detect small-effect loci. Finally, six generations of inbreeding greatly reduced the phenotypic noise owing to segregation within each family.

The IPSRI population will permit replication of defined genotypes and be useful in approaches combining linkage and association mapping with the application of high density markers. Results from a genome-wide scan using 384 simple sequence repeat markers permit preliminary estimates for the degree of divergence among the inbred lines derived from the Illinois protein strains. Analysis of allelic diversity in the inbred protein strains revealed that ~50% of the 384 SSR markers are polymorphic for IHP and ILP, and only 4% showed allele frequency differences among the strains that are consistent with the changes in protein concentration. Using the data for the 500 SNP markers previously genotyped on the progenitor families of the IPSRIs, it was possible to assess population structure and to reduce the original population of 500 to a subset of 138 individuals that effectively represents the phenotypic and allelic variation in the original population (Figure 12.2). This subset of 138 IPSRIs is being used in ongoing mapping studies, which are discussed in greater detail later.

A definitive requirement of subsequent mapping studies that use an advanced random mated population, such as the IPSRIs, is a larger number of molecular markers. The development of low-cost, high-throughput sequencing technologies provides numerous options for IPSRI marker development. A genotyping array for 50,000 maize SNPs was developed for diversity analysis and high density linkage mapping in maize (Ganal *et al.*, 2011). However, marker information may be limited because of the inevitable ascertainment bias that arises with selection of lines for marker development within the array platforms. Because the IPSRIs are derived from nineteenth-century maize germplasm, the lines chosen for SNP arrays may not capture the allelic diversity present

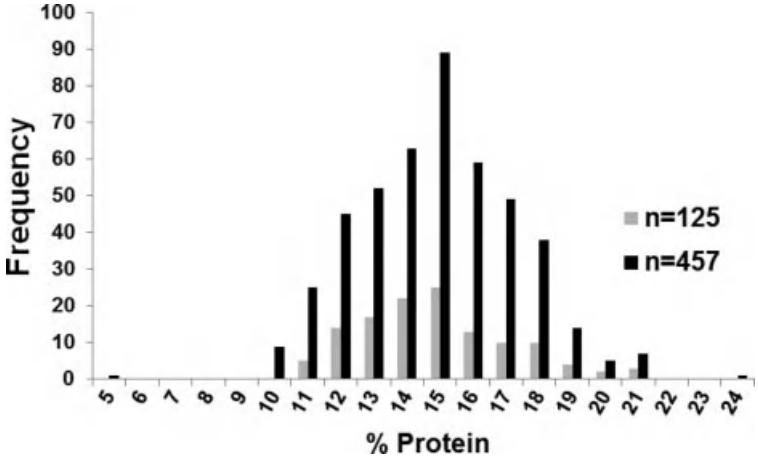

Figure 12.2 Histograms of grain protein concentrations for the larger population of 457 *(black bars)* and reduced set of 138 *(gray bars)* Illinois Protein Strain Recombinant Inbred (IPSRI) lines. Protein concentrations were measured by near-infrared reflectance in 2007.

in the IPSRIs. For example, one use of the 50k SNP array on a population derived from parental lines unrelated to B73 found only 14,000 informative markers. This number could yet be reduced in the IPSRIs. Genotyping by sequencing (GBS), which involves genome complexity reduction using restriction enzymes (Elshire *et al.*, 2011), has been developed more recently and applied to diverse maize populations. GBS of the IPSRIs will likely be useful in assessing patterns of genetic diversity and possibly identify regions with significantly increased LD that could indicate targets of selection, but genetic map construction (Huang *et al.*, 2009) could be problematic depending on how accurately genotypes can be inferred (on the basis of genetic linkage) when not directly sequenced based on genetic linkage.

High density genotyping not only permits mapping experiments but also allows for comparisons of allele frequencies between the strains and generations, such as was done more recently with the Virginia chicken populations divergently selected for body weight (Johansson *et al.*, 2010). Because seeds from previous generations of the selection experiment can be saved and regenerated, genomic comparisons can be made between cycles spanning an even longer time period. Although seeds from the original population are unavailable, seeds are available from cycle 65 and every subsequent cycle thereafter. In this way, comparisons made between cycle 65 and 105 span 40 cycles of selection (Figure 12.1). Comparisons can also be made between the populations at a given time point. The efficacy of GBS for measuring allele frequencies in populations is unknown because read depth needs to increase to call genotypes confidently when more than two alleles are present. The Illinois Selection Strain populations may require the use of sequence capture approaches to sample genetic variation effectively. For example, a recently described method enriches for targeted subsections of the genome and can generate high coverage sequence data at a relatively low cost (Rohland and Reich, 2012). Collectively, the IPSRIs, inbred protein strains, and cycles are invaluable genetic material for various mapping and functional genomics studies.

Characterization of Zein Genes and Their Expression in Illinois Protein Strains

Because zeins can account for 60% of total seed protein in mature grain (Nelson, 1979), it is expected that differences in zein gene organization and expression are associated with the large changes in grain protein concentration observed in the Illinois Protein Strains. Significant differences in the alcohol-soluble fraction of endosperm protein was observed between IHP and ILP in 1922 (Showalter, 1922). Subsequent studies with methods of increasing resolution for individual zein classes and polypeptides confirmed this initial observation (Hansen *et al.*, 1946; Schneider *et al.*, 1952; Lorenzoni *et al.*, 1978; Wilson, 1992; Below *et al.*, 2004) and demonstrated by SDS-PAGE analysis the most dramatic difference in kernel protein accumulation in the Illinois Protein Strains is in the amount of α-zeins produced (Bhattramakki *et al.*, 1996; Below *et al.*, 2004).

Both the 22-kDa and 19-kDa α-zein clusters have been fully sequenced in the B73 inbred, and the 22-kDa cluster has also been sequenced in the BSSS53 inbred (Song *et al.*, 2001), facilitating more detailed studies of zein gene expression. The results of these studies illustrate the dynamic nature of the α-zein genes, which are rapidly evolving in terms of relative chromosomal position, copy number, and expression (see Chapter 8). The zeins are the most highly expressed genes in the endosperm. The 19-kDa and 22-kDa α-zein genes are expressed in a coordinated fashion, beginning around 12 days after pollination (DAP), peaking around 16 DAP, and continuing throughout development, with RNA expression patterns closely matching protein accumulation (Langridge

et al., 1982; Woo *et al.*, 2001). Despite a large copy number, only a fraction of α-zein gene coding sequences produce transcripts, indicating the presence of pseudogenes interspersed with functional genes (Woo *et al.*, 2001; Song and Messing, 2003; Feng *et al.*, 2009). The α-zein genes exhibit variation for the number of expressed zein gene family members among genotypes as well as the relative expression levels of genes shared by genotypes (Feng *et al.*, 2009; Miclaus *et al.*, 2011). Crosses among divergent 22-kDa zein haplotypes produce a range of nonadditive gene expression responses, suggesting ample regulatory variation on which selection for changes in zein accumulation may act.

Global RNA profiling approaches are useful for elucidating genome-wide expression variation that may be contributing to divergent protein phenotypes. A microarray study conducted by our laboratory compared gene expression of developing seeds between the inbred genotypes IHP1 and ILP1. These data are deposited at the NCBI Gene Expression Omnibus (GEO) (accession GSE 18006). Nearly 43,000 probes representing ~30,000 genes showed robust fluorescent signals after data processing and normalization using LIMMA with the R package (Wettenhall and Smyth, 2004). Figure 12.3 plots the expression ratio of IHP1 relative to ILP1 versus average probe signal intensity for this experiment. Following statistical tests for differential expression and Bonferroni correction for false discovery rate, 2.2% of features exhibited robust higher expression in IHP1 compared with ILP1, and 1.6% were more strongly expressed in ILP1. Among the 96 probes annotated as α-zeins on the array (red dots), many exhibit the highest expression levels in the experiment and are strongly upregulated in IHP1. Although also showing relatively strong expression levels, most probes annotated as β-zeins, δ-zeins, and γ-zeins (blue dots) are not differentially expressed between IHP1 and ILP1. All non-zein genes are plotted in gray.

Validation and further analysis are currently ongoing for zein gene expression and other differentially expressed genes that represent candidate targets of long-term selection for grain protein concentration. Owing to its inherent advantages over microarrays for assaying expression of closely related genes and alleles and providing a broader and unbiased survey of gene expression, we have conducted RNASeq experiments to profile gene expression changes in the Illinois Selection strains. Through comparisons of the selected populations at different cycles of the experiment, where multiple alleles and substantial heterozygosity remains, we will more thoroughly document both sequence and regulatory variation in response to long-term selection.

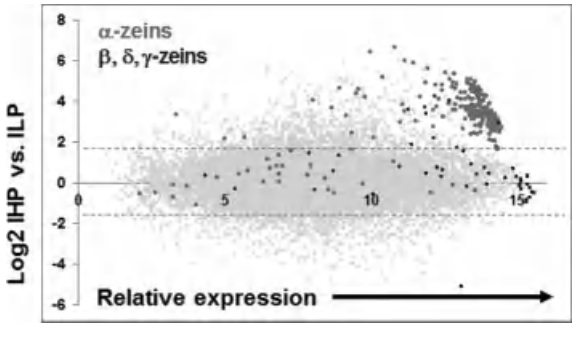

Figure 12.3 Relative zein gene expression levels in 16 DAP seeds as determined by microarray. Plotted is the expression ratio of IHP1 relative to ILP1 versus average probe signal intensity. *Dashed lines* represent cutoffs for features exhibiting statistically significant differential intensity (*t*-test, P value $< .05$; FDR adjusted q value < 0.05). The features annotated as α-zeins are plotted in red, and β-zeins, δ-zeins, and γ-zeins are plotted in blue. All other features are plotted in gray. (For color detail, see color plate section.)

Contribution of Zein Regulatory Factor *Opaque2* to Observed Responses to Selection in Illinois Protein Strains

Both prior QTL mapping studies and RNA profiling experiments suggest that many genes contribute to the dynamic and prolonged responses to selection for grain protein concentrations. However, an alternative hypothesis that cannot yet be excluded is that quantitative variation in the activities of a few key regulatory genes may explain phenotypic changes. A prime candidate for such a regulatory gene is *Opaque2*. *Opaque2* encodes a bZIP transcription factor that activates 22-kDa α-zein gene expression by binding to a highly conserved promoter element (Schmidt *et al.*, 1992). *o2* also interacts with the *Prolamin-box Binding Factor* (*PBF*), a Dof zinc finger DNA binding protein that binds to a highly conserved motif (the prolamin box, 5′-TGTAAAG-3′) found in the promoters of all zein genes and genes encoding prolamins from other cereals (see Chapters 8 and 9) (VicenteCarbajosa *et al.*, 1997; Hwang *et al.*, 2004).

Figure 12.4 shows the phenotypic impacts of introgressing the *o2-R* null mutant allele (Bernard *et al.*, 1994) on seed protein concentrations in the IHP1 and B73 inbred backgrounds. The mutant ear exhibits the characteristic opaque kernel phenotype in the IHP1 background (Figure 12.4*A*). Using estimates from near-infrared reflectance measurements of grain, the *o2-R* mutation reduced total protein from 27.0% to 22.4% in IHP1 and from 8% to 7% in B73. As expected, 22-kDa α-zein protein was significantly reduced in both IHP1 and B73 (Figure 12.4*B*). The 4.6% reduction conditioned by the *o2-R* allele in IHP1 likely represents the maximum possible genetic effect that could be associated with *o2* in the Illinois Protein Strains.

The *o2* locus is highly polymorphic (Henry and Damerval, 1997), and previously developed marker assays for *o2* were used to assess genetic variation at the *o2* locus among the Illinois Protein Strains. Approximately 48 individuals from ILP_{65}, IHP_{65}, ILP_{100}, IHP_{100}, and $IRHP_{100}$ were genotyped using SSR marker umc1066 (maizeGDB), which amplifies the highly polymorphic region of the *o2* gene described by Hartings *et al.* (1994). Polymorphic variants were observed at cycle 65 and 100 in IHP, but all other populations were fixed for a single common variant (data not shown). The two classes of *o2* genotypes in IHP exhibited a significant difference in mean grain protein

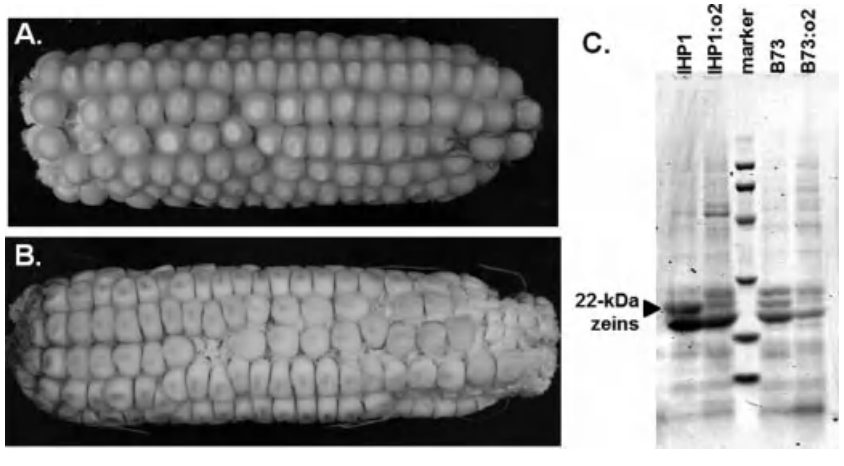

Figure 12.4 Phenotypes of *opaque2* introgressions into IHP1 and B73 inbred lines. *A,* Wild-type IHP1 ear. *B,* IHP1 ear homozygous for the *o2* mutation. *C,* SDS-PAGE of total seed proteins from IHP1, B73, and o2 introgressions into each of these genetic backgrounds. The relative migration of the 22-kDa α-zeins is indicated. (For color detail, see color plate section.)

concentration (32% versus 28%, *t* test $P < .01$). The results of this preliminary experiment suggest that variation at the *Opaque2* locus may contribute to the extreme high protein concentrations in IHP but not the other selected protein strains. Extending such surveys of genetic variation at a genome scale is essential to identify candidate targets of selection versus background genetic drift.

Major Effect QTL May Explain IRHP Phenotype

The dramatic decline of protein concentration in IRHP differs from the steady responses observed in the other protein strains. It took only 15 generations of reverse selection in IRHP to revert nearly 50 generations of gain in IHP, the fastest rate of change in phenotype for any of the selected strains (Figure 12.1*A*). It is likely that this rapid evolutionary change in IRHP may be mediated by variation at relatively few loci with major phenotypic effects. This hypothesis may seem unlikely at first, given the fact that when IRHP was initiated, none of the individuals in cycle 48 of IHP exhibited protein concentrations <15%. However, when considering that the experiment has been conducted using a breeding scheme that deliberately maintains genetic variation and reduces inbreeding, it is unlikely that all alleles that increase grain protein have reached fixation. The continued positive response to selection within IHP (Dudley *et al.* 2007) verifies that alleles favorable to the low protein phenotype remained. We know from the previously described genotyping with SSR markers that IHP1 and IRHP1 are most closely related to each other, sharing alleles at 81% of the marker loci tested compared with the 50% shared between IHP1 and ILP1. It appears that selection in IRHP acted on fewer loci, yet was still rapid and effective.

The low protein phenotype of IRHP differs from that of ILP. Most known mutations that dramatically reduce α-zein accumulation are associated with opaque kernel phenotypes or soft endosperm texture. While ILP kernels also appear opaque, IRHP kernel composition and quality are similar to what is observed following the breeding efforts for Quality Protein Maize where the *opaque2* mutation reduces α-zeins, but modifications in other proteins ameliorate the problem of soft endosperm texture. If relatively few loci regulate the IRHP1 phenotype, identifying these genes may facilitate their use in current elite maize germplasm.

The genetic architecture of the response to selection in IRHP was investigated directly by the generation and evaluation of a population derived from crossing the IRHP1 and IHP1 inbred lines, resulting in a population segregating for protein concentration. The inheritance of protein concentration is well documented to exhibit a strong maternal effect (Tsai *et al.*, 1990). To control for maternal effects, F_1 hybrids between IHP1 and IRHP1 were backcrossed to IRHP1 followed by self-pollination of BC_1 plants, and a population of 115 BC_1S_1 progeny was created. Although the BC_1S_1 ears are either homozygous IRHP1 or heterozygous and segregating in a 1:2:1 ratio, the protein concentration of these ears follows that of the maternal plant. The BC_1S_1 ears segregate similar to the BC_1 plants from which they are derived and are expected at any given locus to segregate in a 1:1 ratio for homozygous IRHP1 versus heterozygous IRHP1/IHP1 genotypes. Grain protein concentrations measured in this population are plotted in a frequency histogram in Figure 12.5. The population is characterized by a bimodal distribution: 51 individuals exhibited <13% protein, with a mean of 10.6% protein, whereas the remaining 64 individuals exhibited protein >13% with a mean of 14.5%. These observations are consistent with equal segregation into two phenotypic classes of greater than or less than 13% protein (χ^2 test 1.47, $P = .22$, df = 1). The bimodal distribution strongly supports the hypothesis of one or a few genes with major effects, with respect to IRHP1. If the trait was controlled by a larger number of genetic factors, a more continuous distribution would be expected with fewer numbers of individuals belonging to more phenotypic classes. The BC_1S_1

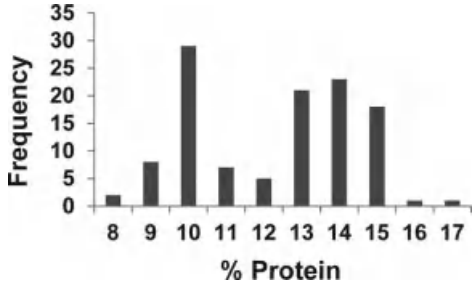

Figure 12.5 Protein concentration frequency histogram of 115 BC_1S_1 individuals from the cross of IHP1 and IRHP1.

population analyzed here represents a good starting point for coarse genetic mapping studies. A larger BC_1S_1 population was generated in summer 2010 for additional fine-mapping. This information will be used to identify gene candidates associated with the more desirable reduced zein phenotype of IRHP1.

Zein Promoter-Reporter Lines to Investigate Regulation of 22-kDa α-Zein Gene Expression in Illinois Protein Strains

The studies summarized in this chapter clearly indicate that the zein synthesis pathway has been one target of selection for altered grain protein concentration in the Illinois Protein Strains. Additional investigations of the molecular mechanisms responsible for these differences may offer insights into various questions related to the control of grain protein concentration. Approaches for studying individual zein gene expression are complicated by the high number and sequence similarity of the α-zein genes and the presence of pseudogenes. Also confounding analysis is the quantitative inheritance of grain protein concentration, where tens to hundreds of genetic factors have been estimated to contribute to the protein concentration differences between IHP and ILP. One approach for tracking expression of individual zein genes is through the use of reporter genes. When fused to a zein gene promoter, the expression of the reporter gene can be measured in a rapid, quantitative, and potentially nondestructive manner to estimate expression of the zein gene of interest.

The most widely used reporter gene for plants is the *GUS* gene. The GUS enzyme has many desirable properties for use as a reporter gene; it can be detected and quantified by histochemical and spectrophotometric assays, is highly sensitive, and does not interfere with normal cellular metabolism (Jefferson *et al.*, 1987). One of the earliest examples of using transgenic promoter-reporter lines to characterize gene expression in maize was the fusion of the *E. coli* β-glucuronidase (*GUS*) reporter gene to a 27-kDa γ-zein promoter (Russell and Fromm, 1997). *GUS* activity was shown to be proportional to transgene RNA expression, inherited in a stable manner, and did not affect endogenous zein gene expression. However, disadvantages of *GUS* are that diffusion of the *GUS* reaction products limits cellular resolution, and assaying *GUS* activity requires destruction of the tissue sample. Fluorescent proteins such as green fluorescent protein (GFP) from jellyfish, modified derivatives of GFP with different spectral properties, and the monomeric red fluorescent protein from reef coral (DsRed) have gained popularity as reporter genes in plants because they overcome some of the limitations associated with the *GUS* reporter gene (Stewart, 2006). Of particular interest for the study of seed gene expression is the ability to visualize the DsRed protein in maize under standard white light, where the kernels appear pink to red in color (Wenck *et al.*, 2003). This property

enables rapid monitoring of gene expression during seed development. Additionally, the relative fluorescent intensity associated with reporter gene expression may be quantified by direct imaging without destruction of the sample.

We have evaluated a collection of transgenic lines produced by other laboratories where zein promoters have been used to drive expression of either the *GUS* or *DsRed* reporter genes. These transgenic lines have been crossed to the Illinois Protein Strain inbreds as well as the reference genotype B73 to assess the impacts of genetic background on the regulation of zein promoter activities. *GUS* reporter lines for the 27-kDa γ-zein and both the 22-kDa and 19-kDa α-zeins were initially obtained from Pioneer Hi-Bred International. The results for the *GUS* lines are described by Salas (2008) and can be briefly summarized as being qualitatively consistent with what is known about the regulation of zein gene expression. GUS staining of the Z27 construct was prevalent throughout the entire endosperm of IHP and ILP, but no variation in the level of expression was observed between them. GUS staining of the Z19 and Z22 constructs was found in the peripheral endosperm, consistent with the findings of Woo *et al.* (2001). As expected, IHP also stained darker than ILP for both the Z19 and the Z22 GUS transgenes.

The fluorescent protein promoter-reporter lines employed here are a part of a series of transgenic reporter maize lines that were created as part of a joint project conducted by Jackson's laboratory at Cold Spring Harbor, Sylvester's laboratory at the University of Wyoming, and the Plant Transformation Center at Iowa State University (Mohanty *et al.*, 2009) (http://maize.tigr.org/cellgenomics/index.shtml). Derived from the coral reef species, *Discosoma,* a monomeric version of the tetrameric DsRed protein termed mRFP1 (Campbell *et al.*, 2002) was fused to the C-terminus of the 22-kDa α-zein gene, *Floury2*. To preserve proper tissue and temporal regulation of the *Floury2*-mRFP1 fusion protein, the construct includes an intact α-zein gene driven by native flanking regulatory elements, including ∼2000 bp of the *Floury2* gene promoter and 1000 bp of 3′ sequence. Inclusion of the native regulatory sequences may allow these lines to provide more information about regulatory elements acting at the level of transcription and elements involved in processing, localization, and turnover of either zein mRNA or protein.

When transformed into a HI-II background, initial analysis of the mRFP kernels by Wenck *et al.* (2003) revealed the ability of the mRFP protein to be visualized under white light, the kernels appearing pink in color. This property can most likely be attributed to the high level of expression of the *Floury2* gene, the relatively high stability of zein proteins, and the use of the monomeric RFP that does not require multimerization to emit fluorescence (Campbell *et al.*, 2002). *Floury2* has been shown to account for ∼20% of all 22-kDa α-zein expression in inbred variety B73 with only one gene expressed more (Song and Messing, 2003; Feng *et al.*, 2009). The ability to visualize *FL2-mRFP* expression under white light allows for the quick nondestructive monitoring of *Floury2* gene expression during seed development.

Regulatory Changes in *FL2-mRFP* Expression When Crossed to Illinois Protein Strains

Three independent *Agrobacterium*-mediated transformation events (47, 52, and 172) for *FL2-mRFP* were used as the donor lines for backcrossing into the four Illinois Protein Strain inbreds and the B73 inbred. Analysis of *FL2-mRFP* expression in the kernels produced from these crosses provides information about the genetic inheritance of the *FL2-mRFP* transgene, its response to genetic background, and its expression throughout development.

Regardless of genetic background, stage of backcrossing, and transgenic event, approximately half of the kernels on an ear are white, and the other half exhibit the red-to-pink coloration associated

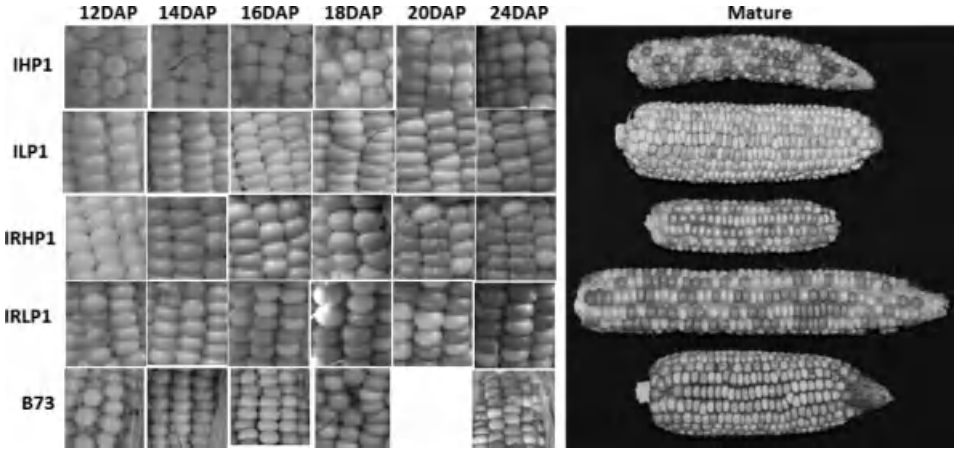

Figure 12.6 Photographs of kernels produced following backcrosses of *FL2-mRFP* transgenic events to the Illinois Protein Strains inbreds (TG event 52) and B73 (TG event 47). Photographs were taken at regular intervals during the period of zein accumulation from 12–24 days after pollination (DAP). (For color detail, see color plate section.)

with the *FL2-mRFP* expression. The 1:1 ratio of white to pink kernels indicates that each transgene is inherited as a single dominant genetic locus. Additionally, *FL2-mRFP* expression is consistent among the three transgenic events, despite having integrated in different locations in the maize genome. This observation demonstrates that location within the tandemly duplicated 22-kDa zein gene cluster is not important for proper regulation of the *FL2-mRFP* reporter gene.

Because the *FL2-mRFP* reporter lines provide a nondestructive visual assay for zein gene expression, transgenic seeds from the crosses to the IPS and B73 were monitored for the onset and progression of red coloration during seed development. Zein accumulation is known to begin at ~10 DAP, peak at ~16 DAP, and continue throughout grain fill. To document *FL2-mRFP* expression throughout development, BC3 kernels from reciprocal crosses of the Illinois Protein Strains and all three transgenic reporter lines were photographed at various time points from 8–24 DAP, where the same ear was used for the entire series of photographs (Figure 12.6). No differences were apparent due to transgenic event or direction of cross. For this reason, only ears from event 52 are shown using the Illinois Protein Strains as the female parents. From these photographs, *FL2-mRFP* expression can be visualized beginning 12 DAP in IHP and IRLP and 14 DAP in ILP, IRHP, and B73 and increases throughout development in all genotypes. Mature ears produced from a similar set of crosses are shown to the right. These results indicate that *FL2-mRFP* expression exhibits spatial and temporal patterns expected for endogenous α-zein genes.

FL2-mRFP expression of BC3 ears also corresponds with known levels of zein protein accumulation. It is the strongest in IHP1, followed by IRHP1, B73, and ILP1. IRLP1 kernels demonstrate much darker pink coloration than is expected given the low protein concentration of this strain. However, because the IRLP1 conversion is not yet complete, it is possible that alleles from the donor parent remain that increase *FL2-mRFP* expression.

To corroborate further mRFP pink coloration intensities with gene expression, zein accumulation and relative expression of zein and *FL2-mRFP* transgene expression using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were examined at 24 DAP in the pink seeds from BC3 ears in IHP1 and ILP1 backgrounds. As expected, zein accumulation was higher in kernels from the IHP1 background compared with ILP1 (Figure 12.7). To determine if gene expression

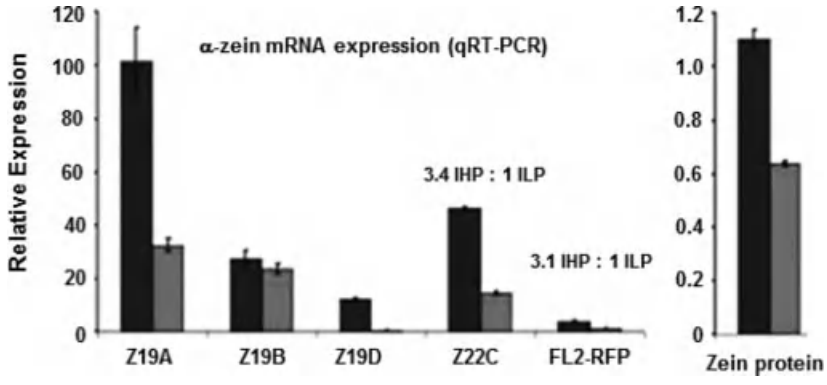

Figure 12.7 Relative expression of the three classes of 19 kDa (z1A, z1B, z1D) and single class of 22 kDa (z1C) α-zein genes in inbred protein strains IHP1 *(black bars)* and ILP1 *(gray bars)* and relative expression of the *FL2-mRFP* transgene when backcrossed (BC3) into IHP1 and ILP1. Total zein protein is also plotted on the right.

patterns correlate with zein protein, primers were designed to anneal to common sequences of each subfamily of the 19-kDa (Z19A, Z19B, and Z19D) and 22-kDa (Z22C) α-zeins and the *FL2-mRFP* transgene, and qRT-PCR was performed. Relative Z19B RNA expression was similar between IHP1 and ILP1, but Z19A, Z19D, and Z22C expression was much higher in IHP1 compared with ILP1. The relative expression of *FL2-mRFP* was also higher in the IHP1 background than ILP1, exhibiting a ratio of (3.1:1). Because *Floury2* belongs to the Z1C subfamily, it is not surprising that Z22C genes were expressed at a similar ratio (3.4:1). These results demonstrate that *FL2-mRFP* protein accumulation follows transgene expression and that *FL2-mRFP* transgene expression is predictive of the entire subfamily of 22-kDa α-zeins. The transgene will not only be useful as a reporter for tracking individual zein gene expression but may represent the whole class of 22-kDa α-zeins.

Regulation of *FL2-mRFP*

As described earlier, the *o2-R* recessive mutant was introgressed to IHP1 to create a near-isogenic line. Here, we use this IHP1: *o2-R* genotype to test whether the *FL2-mRFP* transgene is transcriptionally activated by o2, as are endogenous 22-kDa α-zein genes. Following four backcrosses of the *FL2-mRFP* transgene to IHP1, plants carrying the *FL2-mRFP* transgene were then backcrossed twice to IHP1:*o2-R* to generate ears segregating for both the *FL2-mRFP* transgene and *o2-R*. The resulting ear is shown in Figure 12.8. Kernels homozygous for the *o2-R* mutant allele exhibit the characteristic chalky, opaque kernel phenotype compared with their wild-type counterparts. Approximately 50% of the kernels contain the *FL2-mRFP* transgene, as visualized by the pink coloration of the kernels. Within the transgenic kernels, approximately 25% are also homozygous for *o2-R* and appear both opaque and light pink in color. The reduction in pink coloration indicates decreased *FL2-mRFP* expression and zein protein accumulation. These results demonstrate that the *FL2-mRFP* transgene is regulated by *o2* in a similar fashion as the endogenous *floury2* gene. Collectively, the aforementioned studies indicate that the *FL2-mRFP* transgene behaves like an endogenous zein gene, and the *FL2-mRFP* reporter lines will be a useful tool for further study of 22-kDa α-zein gene regulation.

The *FL2-mRFP* promoter-reporter lines described in this chapter are being used in numerous ongoing genetic experiments. Once fully introgressed into their respective inbred backgrounds,

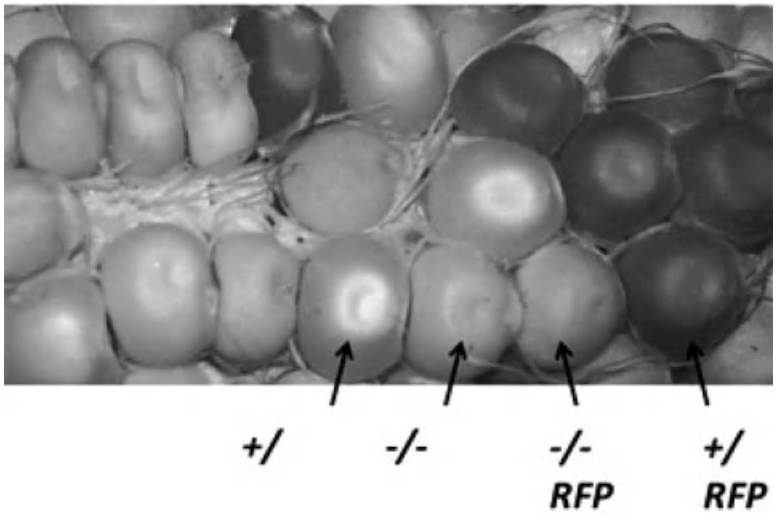

Figure 12.8 Photograph of an IHP1 ear segregating for the *o2* mutation and the *FL2-mRFP* transgene. The *o2* mutant phenotype is chalky and opaque in appearance (−/−) compared with wild-type kernels (+/). Kernels containing both the *o2* mutation and the *FL2-mRFP* transgene (−/−; RFP) illustrate reduced expression of *FL2-mRFP*, as detected by a significant decrease in pink coloration of the kernels compared with kernels containing only the *FL2-mRFP* transgene, which are much darker (+/; RFP). (For color detail, see color plate section.)

the potential impacts of gene dosage and imprinting on the maternal inheritance of grain protein concentration will be investigated through reciprocal crosses. A genetic mapping study is also underway using the *FL2-mRFP* transgene activity as a phenotype. A near-isogenic line of *FL2-mRFP* in the B73 inbred background, which produces grain with ∼7% protein, was crossed to the previously described IPSRI mapping population. The resulting ears demonstrate a wide range of pink coloration. A method for quantification of *FL2-mRFP* expression from high-resolution digital images is in development. The advantages of using *FL2-mRFP* expression as a phenotype is that it specifically tracks regulatory variation for *floury2* 22-kDa α-zeins, rather than seed protein concentration as a whole, which depends on other genetic and environmental factors. Finally, the IHP1:*FL2-mRFP* reporter line has been crossed to a mutagenized (ethyl methanesulfonate) population of IHP1 plants to screen ears visually for mutations with altered *FL2-mRFP* expression and protein concentration. These and other studies combining genomics advances with the Illinois Long Term Selection experiment will continue not only to provide insight into how the maize genome has responded to artificial selection but also may identify genes and regulatory changes that could further improve maize productivity and grain composition.

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13 Machine Vision for Seed Phenomics

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Introduction

Seeds have a broad array of morphological, biochemical, and biological phenotypes. For many crops, the seed is the desired end-product for breeding and cultivation with the seed phenome directly related to product quality. In seed production, it is critical to have viable seeds that germinate and establish well in the field (Aquila, 2009). For seed crops, composition, morphology, and resistance to pathogens are important seed traits that greatly influence the value of a farmer's harvest (Shewry, 2007; Nesi *et al.*, 2008; Fox and Manley, 2009; Pinzi *et al.*, 2009). Most seed traits are complex to score, and destructive analytical approaches are often needed to obtain quantitative data. For example, determining germination rates typically requires a sample from a seed lot to be germinated under controlled conditions and germinated seedlings are scored visually. For biochemical assays such as protein content, a sample of a seed lot is typically ground to a fine powder, dried, and combusted in a C/N analyzer to determine nitrogen content. In these examples, labor-intensive manual counting or seed grinding and chemical analysis is required for quantifying a single seed phenotype.

With the extensive development of genomics technologies, our ability to quantify genetic information and RNA transcripts has become automated with sophisticated computational analysis tools. Even though the seed phenome is related to genetic information, quantifying and studying seed phenotypes is still highly dependent on manual counting and analytics. Systematic and automated phenotyping technologies, also known as phenomics, provide excellent opportunities to align more closely the level of detail in genome and phenotype information to understand better the relationships between genotype and phenotype.

Machine vision approaches are attractive alternatives to traditional phenotyping. Machine vision combines electromagnetic imaging and spectroscopy technologies with computation to replace manual or destructive analytical approaches. Machine vision technologies range from analysis of optical digital images to various types of spectroscopy and nuclear magnetic resonance (NMR) imaging. The types of data that can be extracted from machine vision approaches vary on the wavelengths of light used to image seeds and the preparation of the seed samples for the imaging technology (Figure 13.1). The throughput of these technologies depends on the level of resolution of the images and image acquisition designs. Higher throughput technologies can be automated and provide massive savings in time and cost to obtain quantitative data (Armstrong, 2006). Some machine vision platforms are considered to be complete substitutes for conventional analytical chemistry (Conway and Earle, 1963; Blanco and Villarroya, 2002; Rodriguez-Saona and Allendorf,

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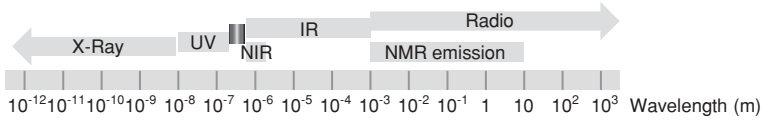

Figure 13.1 Electromagnetic spectrum showing wavelength ranges for machine vision technologies. (For color detail, see color plate section.)

2011). Other imaging technologies provide spatial resolution of quantitative traits that would be impossible to gather without a machine vision platform (Kim *et al.*, 2006).

In this chapter, we highlight machine vision technologies as they have been applied to seed phenotype analyses. The chapter is organized based on spectral type and discusses phenotype features that have been extracted by imaging seeds within each spectral range (Figure 13.1). We start with short-wavelength, high-energy x-rays and end with a discussion of magnetic resonance imaging (MRI), which uses longer wavelength radio waves.

High-Energy Imaging: X-ray Tomography and Fluorescence

The high energy of x-rays allows two types of imaging of biological samples: x-ray attenuation and x-ray fluorescence (Stuppy *et al.*, 2003; Dhondt *et al.*, 2010; Moore *et al.*, 2010; Burghardt *et al.*, 2011). At lower irradiation levels, the differential absorption or attenuation of x-rays by biological tissues can be used to produce radiographs. Similar to laser confocal microscopy, a series of radiographs at serial planes of section can be reconstructed into a three-dimensional image of the sample via computed tomography (CT) scan. The resolution of CT is related to the level of irradiation and focus of the x-ray source. High-powered, monochromatic x-ray sources from synchrotron radiation can be used to obtain three-dimensional images of plant tissues at <1 μm resolution (Stuppy *et al.*, 2003; Dhondt *et al.*, 2010). There are only a few synchrotrons available worldwide for high-resolution x-ray tomography, but laboratory high-resolution CT scanners, commonly known as micro-CT scanners, can also be used for analysis of plant materials. Micro-CT scanners use a polychromatic x-ray source and have lower power translating to slightly lower resolution at similar scanning times between the two methods (Burghardt *et al.*, 2011). In both cases, the level of x-ray exposure needed for high-resolution imaging of seed or plant tissue causes DNA damage, and the technique cannot be considered a live-cell approach. However, CT provides the advantage of being nondestructive; samples can be imaged and then processed for other analytical techniques.

Micro-CT provides many advantages over conventional microscopy. The approach does not require sectioning and can be applied to dry or fossilized samples. Surface images similar to scanning electron micrographs are produced. In addition, samples can be digitally sectioned in any plane once the three-dimensional reconstruction is complete allowing comparison of tomograms with traditional micrographs (Smith *et al.*, 2009). X-ray attenuation is directly related to the density of the sample, and various attenuation cutoffs or thresholds can be used to isolate tissues with similar density and visualize the outer surface, air spaces, or specific structures within the sample. The tomogram can also be used to calculate accurately tissue volume, surface area, porosity, and densities of specific regions within a sample. For density measurements, the polychromatic x-ray sources used in laboratory micro-CT scanners are subject to beam hardening, in which tissues absorb a disproportionate amount of long-wavelength "soft" x-rays leading to errors in the calculated density of thin versus thick samples (Burghardt *et al.*, 2011). The geometry and size of samples need to be similar to compare density measurements directly.

In plants, x-ray tomography has primarily been used to image root systems and vascular systems (Brodersen *et al.*, 2011; Flavel *et al.*, 2012; Hunter *et al.*, 2012). The main application of CT in seeds has been to phenotype fossils for morphological comparison with extant species. In the last 5 years, synchrotron tomography has become an important technique to image the anatomy of small fossilized seed to place plants within anatomically based phylogenies (Friis *et al.*, 2007; Mendes *et al.*, 2008a, 2008b; Chen *et al.*, 2009a, 2009b; Friis *et al.*, 2009). Micro-CT has also been used to study the changes in pore size that occur during coffee bean roasting and to model moisture loss during maize grain drying (Pittia *et al.*, 2011; Takhar *et al.*, 2011). However, the approach has yet to be extensively used for phenotyping live plant seeds even though there is potential for highly quantitative studies. Micro-CT images of maize kernels can distinguish the major anatomical features of a mature kernel: embryo, vitreous endosperm, starchy endosperm, and pericarp (Takhar *et al.*, 2011). Figure 13.2 shows cross sections of micro-CT images of *opaque2* (*o2*) and normal kernels. The *o2* mutant disrupts expression of zein seed storage proteins and starch granule packing with a loss of the hard, vitreous endosperm (see Chapters 8, 9, and 12) (Gibbon and Larkins, 2005).

The hard x-rays from some synchrotron light sources are of sufficient power to complete synchrotron x-ray fluorescence microscopy (SXFM). In SXFM, a monochromatic x-ray beam is focused onto the tissue to eject core shell electrons from the atoms within the sample (Lombi *et al.*, 2011a). When higher shell electrons replace the vacancies, each element emits characteristic wavelengths of light allowing the quantification of biologically relevant ions. Tomography can also be completed with SXFM to produce three-dimensional images of elemental distributions within a tissue. SXFM of barley grains has shown that micronutrients including iron (Fe), copper (Cu), manganese (Mn), potassium (K), and zinc (Zn) all accumulate in localized areas of the seed with the bulk of the accumulation in the embryo and vascular bundle (Lombi *et al.*, 2011b). In *Arabidopsis* seeds, SXFM was used to visualize directly Fe distribution in a mutant of the *VACUOLAR IRON TRANSPORTER 1* (*VIT1*) gene (Kim *et al.*, 2006). Even though *vit1* mutant and wild-type seeds accumulate equivalent

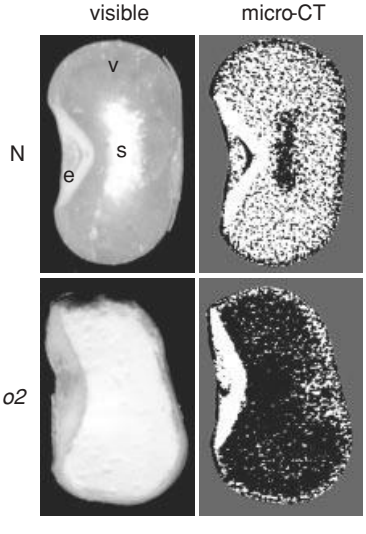

Figure 13.2 Comparison of seed cross sections from normal (N) and *opaque2* (*o2*) mutant. Visible panels are hand sections made after completing CT scans. Micro-CT scans have an identical threshold for attenuation with white pixels indicating regions above the density threshold and black pixels indicating regions below. *v,* vitreous endosperm; *s,* starchy endosperm; *e,* embryo. (For color detail, see color plate section.)

levels of Fe, SXFM tomograms showed that Fe was not as highly localized to provascular regions in *vit1* compared with wild-type.

These examples illustrate how attenuation and fluorescence x-ray CT can yield very high-resolution seed phenotype data that would be difficult to obtain with other analytical techniques. Attenuation provides an alternative to traditional microscopy, whereas x-ray fluorescence provides high resolution of ion concentrations. Equivalent ion quantification technologies require sectioning or dissection of the seed and would not give the same level of three-dimensional resolution (Moore *et al.*, 2010). Data acquisition and processing times are faster for x-ray imaging than traditional microscopy, with scans taking minutes to hours (Dhondt *et al.*, 2010). However, rendering the tomogram is computationally intense and requires significant researcher input. X-ray CT approaches cannot be considered exceptionally high-throughput, and published examples of micro-CT on seeds contain very small sample sizes (Kim *et al.*, 2006; Lombi *et al.*, 2011b; Pittia *et al.*, 2011; Takhar *et al.*, 2011).

Optical Imaging: Visible Spectrum

Optical imaging relies on the transmission and reflectance properties of the visible spectrum. Visible light perceived by the human eye extends from 380–770 nm. The natural resonance frequencies of atoms in a material lead to differential absorption, transmission, and reflectance of the visible spectrum. Chemical bonds influence atomic resonance frequencies leading to some biological compounds having specific visible reflectance such as chlorophylls and carotenoids. Seed color and morphology are very important traits that humans have used to direct crop production either consciously or unconsciously for at least 10,000 years (Purugganan and Fuller, 2009). Seed size, color, and shape remain relevant targets for current breeding strategies as well as genomic studies.

Digital cameras and scanners provide inexpensive image acquisition for machine vision phenotyping of seeds (Dell'Aquila, 2007, 2009). Digital imaging devices convert visible light into electrical signals through proportional conversion of photons into electrons based on the photoelectric effect. Although digital image sensors propagate light intensity information, the sensor does not distinguish the light wavelength. Color is produced by filtering or spectral separation and integrating signals from major color wavelengths: red, green, and blue. A digital camera gives an approximation of the true color in the image. The features from an optical image can be related to a seed's genetic and biological origin, biochemical composition, and potential for future propagation. Currently, digital imaging is used at both commercial and research levels to improve seed phenotyping accuracy and efficiency.

Two important commercial applications of digital imaging include seed identification and testing seed germination. Seed lots are graded based on grain quality and manually inspected for the presence of potentially noxious weed species (USDA, 2009). Automated image-based platforms are being tested as alternatives for manual assessments. Seed identification projects have found that multivariate mathematical models can be trained to identify weed seeds accurately by using data extracted from images of the seed (Chtioui *et al.*, 1996; Granitto *et al.*, 2005). In one example, a seed identification system identified ~98% of weed seeds from 236 different species by using an optimized set of just four image features (Granitto *et al.*, 2005). Seed identification is also important for determining the final grade and price of grain lots. For example, wheat grain lots are graded by class and subclass. Machine vision approaches have been tested for their ability to discriminate between different wheat grain classes and similar species such as rye, barley, and oats (Majumdar and Jayas, 2000a, 2000b, 2000c, 2000d; Paliwal *et al.*, 2003; Arefi *et al.*, 2011; Mebatsion *et al.*,

2012). These CCD camera-based platforms can be 95%–100% accurate at identifying similar grains. Accuracy improves as mathematically derived features such as Hue moment invariants and Fourier transformations are included in the feature set. The images can also be used to identify damaged grain and foreign material, each of which is important in assigning the final grade of the seed lot (Paliwal *et al.*, 2003).

Seedling establishment and vigor in the field is an important trait for yield. Uniform establishment reduces weed pressure, allows for reduced application of inputs, and promotes more uniform flowering and harvest (Ashraf and Foolad, 2005). Establishment is correlated with the speed, proportion, and uniformity of seed germination under stressful environments, and seed germination assays involving multiple stressful environments are a standard approach to characterizing seed lot quality (Bingham *et al.*, 1994). Germination assays either count seedlings during a predetermined timeframe for a mean germination time or count root radical emergence, which is an earlier phase of germination (Matthews *et al.*, 2011). Machine vision platforms using digital cameras or flatbed scanners can capture both types of germination scores, but root radical emergence is more amenable to machine vision approaches.

The French National Seed Testing Facility (Algers, France) runs a semiautomated seed phenotyping platform using Jacobsen tables for environmental control with four static cameras per table (Wagner *et al.*, 2011). Computer control systems dictate periodic image acquisition, and because samples are imaged within the environment in which they are grown, individual seed germination profiles can be traced from imbibition to early seedling stage. Basic measurements such as area and XY position are automatically extracted from each seed by the image processing program ImageJ. This facility has imaged >170,000 seeds from plants such as tomato, alfalfa, sunflower, garlic, and maize since 2005 (Ducournau *et al.*, 2005; Wagner *et al.*, 2011). Similar machine vision approaches have been developed for melon and lettuce seed vigor tests (Sako *et al.*, 2001; Marcos *et al.*, 2006).

Germination assays for very small seeds such as *Arabidopsis* are also possible (Joosen *et al.*, 2010, 2012). In this case, seeds are germinated in clear plastic trays on blue filter paper, and the trays are moved from the growth chamber to the camera for each image. Automated image processing extracts multiple germination parameters such as maximum germination and area under the germination curve. The platform was used to phenotype *Arabidopsis* recombinant inbred lines (RILs) for quantitative trait loci (QTL) germination under salt stress. This study found two QTL for maximum germination under salt stress that overlapped with prior studies as well as novel QTL (Quesada *et al.*, 2002; Clerkx *et al.*, 2004; Ren *et al.*, 2010). By completing the phenotyping with a machine vision platform, multiple traits such as time to 50% germination and area under the germination curve could be scored for QTL mapping.

Machine vision platforms can also be used to characterize seed morphology. In maize, kernel hardness and flour quality are associated with the proportion of the kernel that is vitreous. Vitreousness is a qualitative trait that is difficult to score and is traditionally scored by eye using a light box (Fox and Manley, 2009). In an effort to make this trait more quantitative, Erasmus and Taylor (2004) developed an optical imaging system to measure visible light attenuation through maize kernels. The calibration to physically dissected kernels suggested a correlation that could roughly sort kernels by vitreousness. A similar approach has been used to phenotype Quality Protein Maize RILs to map modifiers of the *o2* mutant (Holding *et al.*, 2008, 2011).

In wheat, both a larger grain size and a more spherical shape are considered desirable characteristics for the kernel (Evers *et al.*, 1990). A commercialized digital camera system (MARVIN seed analyzer) was recently used to characterize the underlying genetic framework of wheat grain morphology (Gegas *et al.*, 2010). Morphological features for size and shape were extracted from six diverse, recombinant doubled-haploid populations as well as primitive and modern elite varieties.

The dataset was used to describe the morphological variability and relationships within a broad wheat population and identify QTL associated with agronomic traits. Grain size and shape were found to be largely independent features, and the genetic structure for determining grain size was quite variable between the different germplasm sources. These observations suggest great potential for selecting grain size and shape as independent traits in wheat and illustrate how machine vision platforms can complete thousands of meticulous measurements through image analysis algorithms.

Flatbed scanners provide high-resolution images at low cost making them an effective tool for imaging small seeds. *Arabidopsis* seed areas can be accurately extracted from scanner images allowing large-scale screens for seed size (Herridge *et al.*, 2011). By examining two RIL populations, Herridge *et al.* (2011) found four and five QTL that explained 43% and 44% of the total seed weight variation. Only one common QTL was identified showing that *Arabidopsis* accessions control seed size with multiple genetic pathways. Subtle seed size mutants were also detected with one mutant having only a 10% reduced seed area. Using a similar flatbed scanner imaging system, *Arabidopsis* seed size variation has been related to seedling root growth to identify a direct physiological relationship between seed size and seedling establishment that is independent of genetic variation (Brooks *et al.*, 2010).

So far, optical imaging of seeds has primarily been used to score seeds for traits of interest. Key advantages of machine vision scoring are higher throughput and the ability to score multiple traits from single images using image processing algorithms. In several examples, optical imaging of seeds has enabled discovery of complex relationships and condition-specific QTL that were practically possible only through implementation of a machine vision approach (Brooks *et al.*, 2010; Gegas *et al.*, 2010; Herridge *et al.*, 2011; Joosen *et al.*, 2012). Machine vision based on optical imaging is not yet used extensively for selecting seeds with desired morphology. However, visible wavelengths are frequently incorporated with near-infrared (NIR) spectroscopy to remove undesirable seeds or to select for seeds of interest (Osborne, 2006).

Resonance Absorption: Infrared Spectrum

The infrared (IR) spectrum has lower energy than visible light. Chemical bonds absorb IR light to alter the vibrations and rotations between the atoms within a molecule. The bonds within organic molecules have characteristic resonance frequencies that absorb specific light wavelengths (Rodriguez-Saona and Allendorf, 2011). Resonance frequencies are typically within the mid-infrared (mid-IR) from 2500–25,000 nm. The same bonds can absorb at overtone frequencies, which are shorter wavelengths that are multiples of the resonance frequencies. The overtone wavelengths are close to the visible spectrum (750–2500 nm) and termed NIR. Although molecules absorb mid-IR and NIR based on the same chemical properties, the energy inherent to the light spectrum significantly changes the spectroscopy apparatus and potential applications in seed phenotyping.

Mid-IR is more readily absorbed by biological macromolecules. At the same intensity of mid-IR and NIR light, the mid-IR does not penetrate as thick of a sample (Rodriguez-Saona and Allendorf, 2011). Mid-IR is typically used as an analytical chemistry technique for thin samples to identify spectral fingerprints and chemical composition. Many mid-IR applications focus on food processing rather than seed phenotyping. Mid-IR spectral fingerprints can be used to differentiate milling fractions of wheat and to track protein structural changes during soybean heat processing (Barron, 2011; Samadi and Yu, 2011). With high-energy synchrotron light sources, mid-IR can be used to characterize thin sections of seeds to identify compositional differences between tissue layers in both soybean and maize seeds (Yu *et al.*, 2004; Pietrzak and Miller, 2005).

NIR is not as readily absorbed, and thicker samples are needed to obtain spectra. For seeds, NIR data typically are collected on ground meal or bulk samples of whole seeds. However, single-seed hyperspectral imaging is also possible (Manley *et al.*, 2009; Zhu *et al.*, 2011). NIR spectra have broad absorption peaks and do not provide a specific fingerprint of chemical composition (Osborne, 2006; Cozzolino, 2009). To correlate the spectra to specific chemical composition, multivariate statistical approaches known as chemometrics are used (Blanco and Villarroya, 2002). Chemometrics is an empirical approach in which spectra are collected on a set of test samples that are quantified with other analytical techniques. The spectral and analytical data are related to each other using multiple linear regression, principal component regression, or partial least-squares regression. Artificial neural networks are also sometimes used for relating IR spectral data to chemical composition. Chemometric calibrations are made by collecting spectra from samples followed by determining their composition with reference analytical methods. NIR predictions contain error from both a reference method and spectra collection and cannot be as accurate as the reference method.

Even with this caveat, NIR has been widely adopted from research laboratories to grain elevators to predict seed traits (Osborne, 2006). In maize, calibrations exist for multiple seed constituents, including protein, oil, starch, and moisture (Orman and Schuman, 1991; Berardo *et al.*, 2009); fatty acids (Yang *et al.*, 2009); amino acids (Fontaine *et al.*, 2002); carotenoids (Brenna and Berardo, 2004); and amylose-to-amylopectin ratios (Campbell *et al.*, 1997, 1999, 2002). More complex grain quality traits are correlated with chemical composition, and NIR has been used to predict wet milling starch yields (Paulsen *et al.*, 2003a, 2003b; Paulsen and Singh, 2004), kernel hardness (Robutti, 1995; Correa *et al.*, 2002; Ngonyamo-Majee *et al.*, 2008), and fungal infection (Pearson *et al.*, 2001; Dowell *et al.*, 2002). Although many of these calibrations have been developed using different NIR spectrometers and wavelength regions, these studies demonstrate a wide range of traits that could be estimated from a single NIR reading. The ability to predict multiple seed phenotypes with a single, nondestructive spectroscopic measurement provides massive cost savings over reference analytical methods that are usually limited to a small number of traits. More recently, bulk NIR was used to phenotype the maize Nested Association Mapping panel for kernel composition (Cook *et al.*, 2012). This collection of 25 RIL populations identified candidate genes that control seed composition and included genes known to affect protein, starch, or oil accumulation along with hundreds of new candidates. The chemical phenotypes of the >60,000 samples would not have been practical to score without NIR spectroscopy.

NIR spectroscopy can also be applied to single seeds. Conventional ground and whole-grain NIR use multiple seeds to estimate average composition for the seed sample. In segregating populations, seeds with different composition are averaged, reducing the ability to discriminate for quality traits. Single-seed selections allow more rapid breeding gain or can be used to sort seeds for phenotypes of interest such as protein quantity in soybean or spinach seed viability (Lee *et al.*, 2010; Olesen *et al.*, 2011). Single-seed NIR has been most successful for predicting oil and protein content in seeds with relatively uniform distribution of constituents, such as rapeseed (Velasco *et al.*, 1999a; Velasco and Mollers, 2002; Hom *et al.*, 2007; Niewitetzki *et al.*, 2010), wheat (Delwiche *et al.*, 1998; Delwiche and Hruschka, 2000) and sunflower achenes (Velasco *et al.*, 1999b, 2004). More recently, single-seed calibrations have been developed for oil quality in oilseed rape (Niewitetzki *et al.*, 2010).

Single-seed NIR has been more challenging for large seeded crops such as maize. Maize kernels have large embryos resulting in an asymmetrical distribution of protein, starch, and oil. The asymmetry gives distinct NIR spectra when the germinal or abgerminal side of the kernel is presented to the spectrometer (Orman and Schumann, 1992; Weinstock *et al.*, 2006; Janni *et al.*, 2008). Maize kernels are also variable in size and shape and present differing surface areas and path lengths.

Transmittance spectroscopy can account for asymmetries by collecting spectra from light passing through whole kernels. However, attempts to predict moisture and oil using single-kernel transmittance have had mixed success (Finney and Norris, 1978; Orman and Schumann, 1992; Cogdill *et al.*, 2004).

An alternative approach is to obtain consistent reflectance NIR spectra such as spectra collected specifically from the abgerminal side of the kernel or hyperspectral imaging in which NIR data are selected from relevant sections of the kernel (Baye *et al.*, 2006; Weinstock *et al.*, 2006). Although these approaches yield acceptable predictions, they are low throughput. The kernels need to be hand-placed to ensure a specific kernel orientation or spacing. Spectral acquisition systems that obtain average reflectance over the whole kernel surface have been shown to be robust and have much greater throughput (Armstrong, 2006; Janni *et al.*, 2008). The Armstrong (2006) single-seed NIR system has been used to survey natural variation of maize and common bean (Spielbauer *et al.*, 2009; Hacisalihoglu *et al.*, 2010). The primary innovations that enable high-throughput NIR data collection are the use of an indium-gallium-arsenide (InGaAs) spectrometer with a fast integration time and a tube that illuminates a seed and captures a spectrum while it falls through the tube (Figure 13.3). Calibrations for this single-seed apparatus have been developed for protein, oil, starch, and seed weight for soybean, maize, and common bean indicating that single-seed NIR is practical for large-seeded crops (Armstrong, 2006; Spielbauer *et al.*, 2009; Tallada *et al.*, 2009; Hacisalihoglu *et al.*, 2010).

In contrast to micro-CT and optical imaging, mid-IR and NIR imaging technologies provide information on chemical composition of seeds. NIR is very amenable to developing high-throughput assays for seed sorting or composition but requires chemometric approaches to predict seed composition. By contrast, mid-IR spectroscopy and microscopy provide more direct information regarding chemical composition but can be used only on thin sections or ground seed material. NIR is generally limited to predicting the major macromolecules within seeds, but minor constituents or nonorganic molecules can be predicted if they have strong correlation with major components.

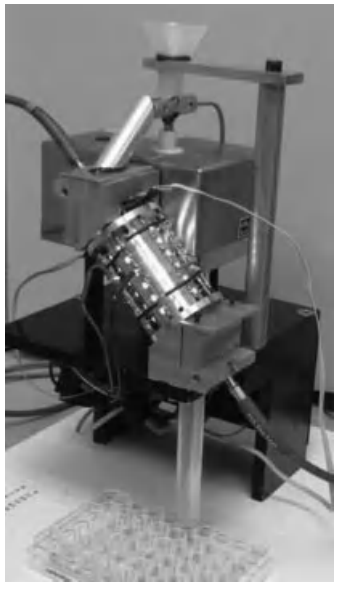

Figure 13.3 Armstrong (2006) single-kernel grain analyzer collects individual seed weight on a microbalance and then NIR reflectance spectrum as a seed falls through the illuminated light tube.

Resonance Emission: Nuclear Magnetic Resonance

NMR spectroscopy and MRI use strong magnetic fields to polarize the nuclear spin on specific isotopes (Kockenberger *et al.*, 2004; Van As, 2007). Magnetized isotopes can absorb and re-emit radiofrequency pulses at their specific resonance frequencies. In biological samples, NMR typically measures ^{1}H or ^{13}C isotopes. Chemical bonds between H and C atoms influence nuclear spin and broaden the NMR resonance frequency, reducing the signal and increasing noise. When molecules are in a fluid state, the anisotropic interactions in chemical bonds are averaged out, and NMR signals can be detected for liquids, such as water or lipid, within biological samples. Crystalline and globular macromolecules such as cellulose, starch, or protein are not easily detected. ^{1}H NMR generally reports the water within developing seeds and lipid storage bodies in a mature, dry seed. The natural abundance of ^{13}C is low, and ^{13}C NMR requires labeling with specific molecules enriched for high ^{13}C content.

In seeds, ^{13}C NMR is primarily used for metabolic flux analysis as a diagnostic and analytic tool (Eisnereich and Bacher, 2007). NMR spectroscopy identifies the specific location of ^{13}C atoms within purified metabolites after a labeling period. NMR spectroscopy data are also quantitative, yielding estimates of the relative amounts of the isotope variants of a metabolite. Combined, ^{13}C-labeling data can be used to infer the enzymatic reactions that most likely gave rise to the isotope combinations observed for a metabolite. Similar to mid-IR spectroscopy, NMR flux analysis requires destructive sampling and intense researcher input for interpretation, placing this approach outside of machine vision strategies.

In contrast, direct ^{1}H NMR spectroscopy measurements can be made to quantify total oil or moisture content in seeds (Conway and Earle, 1963). Oil measurements are possible on seeds from various plants including maize and soybean (Alexander *et al.*, 1967; Collins *et al.*, 1967). The accuracy and repeatability of NMR oil measurements are so high that NMR is a standard method for measuring oil content of mature seeds. NMR measurements are routine for assessing seed oil contents to screen diverse germplasm (Wang *et al.*, 2010, 2011; Horn *et al.*, 2011), for mapping seed oil QTL (Song *et al.*, 2004), and to characterize transgenic germplasm (Shen *et al.*, 2010). Maize seed NMR has been used successfully to clone a major QTL for oil accumulation, which encodes a type I acyl-CoA:diacylglycerol acyltransferase or DGAT1 (Zheng *et al.*, 2008). NMR oil measurements are also very accurate at the single seed level, and single-seed NMR has been successfully used to increase genetic gain in selections for high oil maize lines (Alexander, 1982; Pamin *et al.*, 1986; Song *et al.*, 1999).

MRI of seeds can yield two-dimensional or three-dimensional images for quantitative information about the water and oil distribution within the seed. Two-dimensional MRI has been used to follow moisture loss during wheat kernel drying (Ghosh *et al.*, 2006), and in recent years, MRI has been applied to living, developing seeds to follow oil deposition in barley and endosperm development in pea (Neuberger *et al.*, 2008, 2009). When the developing endosperm is in a liquid state, it is possible to resolve peaks for major metabolites in three dimensions (Neuberger *et al.*, 2009). By engineering custom double coils to detect both ^{1}H and ^{13}C isotopes and pulse labeling of developing seeds, the uptake and metabolism of ^{13}C-sucrose was imaged in developing barley seeds (Melkus *et al.*, 2011; Rolletschek *et al.*, 2011). These live seed imaging approaches open the possibility for developing much more accurate metabolic flux maps that incorporate the movement of metabolites within seed tissues.

In many ways, NMR and x-ray imaging are comparable. Both technologies allow high-resolution tomography and quantification of the chemical composition of a seed. However, the technologies are most appropriate for very different types of samples. NMR detects chemical compounds, whereas x-ray fluorescence detects elements. NMR images and quantifies chemicals in a liquid state, whereas

micro-CT needs significant density contrast within the sample to visualize internal structures effectively. NMR spectroscopy also shares some common features to NIR predictions. Similar to NIR, NMR oil measurements are fast and can be completed with minimal sample preparations at various scales down to single seeds. However, NMR is more limited in the chemical phenotypes that can be determined from mature, dry seeds.

Conclusion

Characterizing the seed phenome is one of the major challenges to developing a comprehensive understanding of gene function in plants. Machine vision technologies are a rich resource for characterizing seed phenotypes and defining the phenotype space for a species. By using spectroscopy and electromagnetic radiation–based imaging, machine vision technologies provide quantitative data about the seed phenome. A key advantage of machine vision is the ability to reanalyze or calculate new phenotypes from pre-existing raw images or spectroscopy data. For example, new predictions can be made from pre-existing NIR data as new calibrations become available. In contrast, manual measurements or chemical assays are usually limited to the specific measurements made at the time by the investigator. There is no single machine vision technology that can provide both structural and chemical information about plant seeds at all stages of development. The decision to use a particular technology and portion of the electromagnetic radiation spectrum is made based on the traits that are viewed as important for the process that is under study. Many of these technologies are nondestructive or minimally invasive and can be used in series to gain a more complete quantification of a seed phenotype. Combining these quantitative phenotype measures with genomics information should help elucidate gene function within the seed.

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